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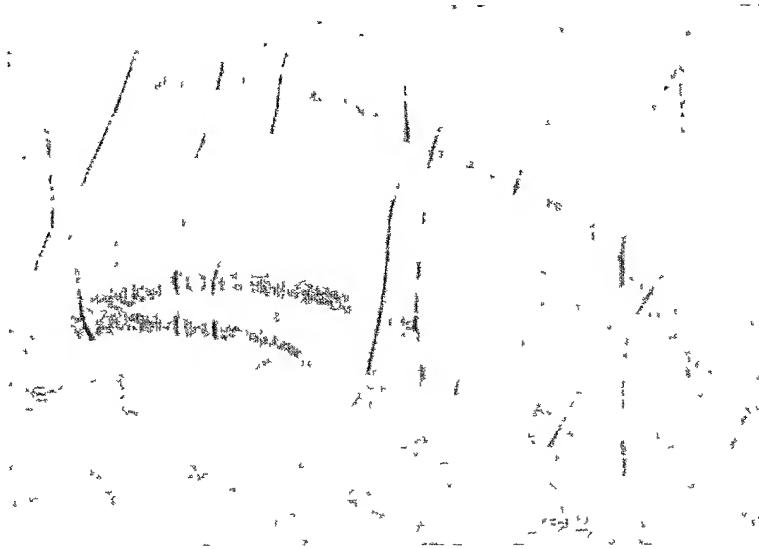
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(54) Title: NOVEL OLIGONUCLEOTIDE COMPOSITIONS AND PROBE SEQUENCES USEFUL FOR DETECTION AND ANALYSIS OF microRNAs AND THEIR TARGET mRNAs



(57) Abstract: The invention relates to ribonucleic acids and oligonucleotide probes useful for detection and analysis of microRNAs and their target mRNAs, as well as small interfering RNAs (siRNAs)

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NOVEL OLIGONUCLEOTIDE COMPOSITIONS AND PROBE SEQUENCES USEFUL FOR DETECTION AND ANALYSIS OF  
MICRORNAs AND THEIR TARGET MRNAs

The present invention relates to ribonucleic acids and oligonucleotide probes useful for detection and analysis of microRNAs and their target mRNAs, as well as small interfering

5 RNAs (siRNAs). The invention furthermore relates to oligonucleotide probes for detection and analysis of other non-coding RNAs, as well as mRNAs, mRNA splice variants, allelic variants of single transcripts, mutations, deletions, or duplications of particular exons in transcripts, e.g. alterations associated with human disease, such as cancer.

Background of the Invention

10 The present invention relates to the detection and analysis of target nucleotide sequences in a wide variety of nucleic acid samples and more specifically to the methods employing the design and use of oligonucleotide probes that are useful for detecting and analysing target nucleotide sequences, especially RNA target sequences, such as microRNAs and their target mRNAs and siRNA sequences of interest and for detecting differences between nucleic acid 15 samples (e.g., such as samples from a cancer patient and a healthy patient).

*MicroRNAs*

The expanding inventory of international sequence databases and the concomitant sequencing of more than 200 genomes representing all three domains of life - bacteria, archaea and eukaryota - have been the primary drivers in the process of deconstructing living 20 organisms into comprehensive molecular catalogs of genes, transcripts and proteins. The importance of the genetic variation within a single species has become apparent, extending beyond the completion of genetic blueprints of several important genomes, culminating in the publication of the working draft of the human genome sequence in 2001 (Lander, Linton, Birren *et al.*, 2001 *Nature* 409: 860-921; Venter, Adams, Myers *et al.*, 2001 *Science* 291: 25 1304-1351; Sachidanandam, Weissman, Schmidt *et al.*, 2001 *Nature* 409: 928-933). On the other hand, the increasing number of detailed, large-scale molecular analyses of transcription originating from the human and mouse genomes along with the recent identification of several types of non-protein-coding RNAs, such as small nucleolar RNAs, siRNAs, microRNAs and antisense RNAs, indicate that the transcriptomes of higher eukaryotes are much more 30 complex than originally anticipated (Wong *et al.* 2001, *Genome Research* 11: 1975-1977; Kampa *et al.* 2004, *Genome Research* 14: 331-342).

As a result of the Central Dogma: 'DNA makes RNA, and RNA makes protein', RNAs have been considered as simple molecules that just translate the genetic information into protein.

Recently, it has been estimated that although most of the genome is transcribed, almost 97% of the genome does not encode proteins in higher eukaryotes, but putative, non-coding RNAs (Wong *et al.* 2001, *Genome Research* 11: 1975-1977). The non-coding RNAs (ncRNAs) appear to be particularly well suited for regulatory roles that require highly specific nucleic acid recognition. Therefore, the view of RNA is rapidly changing from the merely informational molecule to comprise a wide variety of structural, informational and catalytic molecules in the cell.

Recently, a large number of small non-coding RNA genes have been identified and designated as microRNAs (miRNAs) (for review, see Ke *et al.* 2003, *Curr.Opin. Chem. Biol.* 7:516-523). The first miRNAs to be discovered were the lin-4 and let-7 that are heterochronic switching genes essential for the normal temporal control of diverse developmental events (Lee *et al.* 1993, *Cell* 75:843-854; Reinhart *et al.* 2000, *Nature* 403: 901-906) in the roundworm *C. elegans*. miRNAs have been evolutionarily conserved over a wide range of species and exhibit diversity in expression profiles, suggesting that they occupy a wide variety of regulatory functions and exert significant effects on cell growth and development (Ke *et al.* 2003, *Curr.Opin. Chem. Biol.* 7:516-523). Recent work has shown that miRNAs can regulate gene expression at many levels, representing a novel gene regulatory mechanism and supporting the idea that RNA is capable of performing similar regulatory roles as proteins. Understanding this RNA-based regulation will help us to understand the complexity of the genome in higher eukaryotes as well as understand the complex gene regulatory networks.

miRNAs are 18-25 nucleotide (nt) RNAs that are processed from longer endogenous hairpin transcripts (Ambros *et al.* 2003, *RNA* 9: 277-279). To date more than 1420 microRNAs have been identified in humans, worms, fruit flies and plants according to the miRNA registry database release 5.1 in December 2004, hosted by Sanger Institute, UK, and many miRNAs that correspond to putative genes have also been identified. Some miRNAs have multiple loci in the genome (Reinhart *et al.* 2002, *Genes Dev.* 16: 1616-1626) and occasionally, several miRNA genes are arranged in tandem clusters (Lagos-Quintana *et al.* 2001, *Science* 294: 853-858). The fact that many of the miRNAs reported to date have been isolated just once suggests that many new miRNAs will be discovered in the future. A recent in-depth transcriptional analysis of the human chromosomes 21 and 22 found that 49% of the observed transcription was outside of any known annotation, and furthermore, that these novel transcripts were both coding and non-coding RNAs (Kampa *et al.* 2004, *Genome Research* 14: 331-342). Another recent paper describes the use of phylogenetic shadowing profiles to predict 976 novel candidate miRNA genes in the human genome (Berezikov *et al.* 2005, *Cell* 120: 21-24) from whole-genome human/mouse and human/rat augments. Most of the candidate miRNA genes were found to be conserved in other vertebrates, including dog,

cow, chicken, opossum and zebrafish. Thus, the identified miRNAs to date represent most likely the tip of the iceberg, and the number of miRNAs might turn out to be very large.

The combined characteristics of microRNAs characterized to date (Ke *et al.* 2003, *Curr.Opin. Chem. Biol.* 7:516-523; Lee *et al.* 1993, *Cell* 75:843-854; Reinhart *et al.* 2000, *Nature* 403:

5 can be summarized as:

1. miRNAs are single-stranded RNAs of about 18-25 nt that regulate the expression of complementary messenger RNAs

2. They are cleaved from a longer endogenous double-stranded hairpin precursor by the enzyme Dicer.

10 3. miRNAs match precisely the genomic regions that can potentially encode precursor miRNAs in the form of double-stranded hairpins.

4. miRNAs and their predicted precursor secondary structures may be phylogenetically conserved.

Several lines of evidence suggest that the enzymes Dicer and Argonaute are crucial

15 participants in miRNA biosynthesis, maturation and function (Grishok *et al.* 2001, *Cell* 106: 23-24). Mutations in genes required for miRNA biosynthesis lead to genetic developmental

defects, which are, at least in part, derived from the role of generating miRNAs. The current view is that miRNAs are cleaved by Dicer from the hairpin precursor in the form of duplex, initially with 2 or 3 nt overhangs in the 3' ends, and are termed pre-miRNAs. Cofactors join

20 the pre-miRNP (microRNA RiboNucleoProtein- complexes) and unwind the pre-miRNAs into single-stranded miRNAs, and pre-miRNP is then transformed to miRNP. miRNAs can recognize regulatory targets while part of the miRNP complex. There are several similarities between miRNP and the RNA-induced silencing complex, RISC, including similar sizes and both containing RNA helicase and the PPD proteins. It has therefore been proposed that

25 miRNP and RISC are the same RNP with multiple functions (Ke *et al.* 2003, *Curr.Opin. Chem. Biol.* 7:516-523). Different effectors direct miRNAs into diverse pathways. The structure of pre-miRNAs is consistent with the observation that 22 nt RNA duplexes with 2 or 3 nt overhangs at the 3' ends are beneficial for reconstitution of the protein complex and might be required for high affinity binding of the short RNA duplex to the protein components (for

30 review, see Ke *et al.* 2003, *Curr.Opin. Chem. Biol.* 7:516-523).

Growing evidence suggests that miRNAs play crucial roles in eukaryotic gene regulation. The first miRNAs genes to be discovered, lin-4 and let-7, base-pair incompletely to repeated

elements in the 3' untranslated regions (UTRs) of other heterochrony genes, and regulate the translation directly and negatively by antisense RNA-RNA interaction (Lee *et al.* 1993, Cell 75:843-854; Reinhart *et al.* 2000, Nature 403: 901-906). Other miRNAs are thought to interact with target mRNAs by limited complementary and suppressed translation as well

5 (Lagos-Quintana *et al.* 2001, Science 294: 853-858; Lee and Ambros 2001, Science 294: 858-862). Many studies have shown, however, that given a perfect complementarity between miRNAs and their target RNA, could lead to target RNA degradation rather than inhibit translation (Hutvagner and Zamore 2002, Science 297: 2056-2060), suggesting that the degree of complementarity determines their functions. By identifying sequences with near

10 complementarity, several targets have been predicted, most of which appear to be potential transcriptional factors that are crucial in cell growth and development. The high percentage of predicted miRNA targets acting as developmental regulators and the conservation of target sites suggest that miRNAs are involved in a wide range of organism development and behaviour and cell fate decisions (for review, see Ke *et al.* 2003, Curr.Opin. Chem. Biol. 7:516-523). For example, John *et al.* 2004 (PLoS Biology 2: e363) used known mammalian

15 miRNAs to scan the 3' untranslated regions (UTRs) from human, mouse and rat genomes for potential miRNA target sites using a scanning algorithm based on sequence complementarity between the mature miRNA and the target site, binding energy of the miRNA: mRNA duplex and evolutionary conservation. They identified a total of 2307 target mRNAs conserved

20 across the mammals with more than one target site at 90% conservation of target site sequence and 660 target genes at 100% conservation level. Scanning of the two fish genomes; *Danio rerio* (zebrafish) and *Fugu rubripes* (Fugu) identified 1000 target genes with two or more conserved miRNA sites between the two fish species (John *et al.* 2004 PLoS

25 Biology 2: e363). Among the predicted targets, particularly interesting groups included mRNA encoding transcription factors, components of the miRNA machinery, other proteins involved in the translational regulation as well as components of the ubiquitin machinery. In a recent paper, Lewis *et al.* (Lewis *et al.* 2005, Cell 120: 15-20) predicted regulatory mRNA targets of vertebrate microRNAs by identifying conserved complementarity to the so-called seed

30 (comprising nucleotides 2 to 7) sequence of the miRNAs. In a comparative four-genome analysis of all the 3' UTRs, ca. 5300 human genes were implicated as miRNA targets, which represented ca 30% of the gene set used in the analysis. In another recent publication, Lim *et al.* (Lim *et al.* 2005, Nature 433: 769-773) showed that transfection of HeLa cells with miR-124, a brain-specific microRNA, caused the expression profile of the HeLa cells to shift towards that of brain, as revealed by genome-wide expression profiling of the HeLa mRNA

35 pool. By comparison, delivery of miR-1 to the HeLa cells shifted the mRNA profile toward muscle, the tissue where miR-1 is preferentially expressed. Lim *et al.* (Lim *et al.* 2005, Nature 433: 769-773) subsequently showed that the 3' un-translated regions of the downregulated mRNAs had a significant propensity to pair to the seed sequence of the 5' end of the two miRNAs, thus implying that metazoan miRNAs can reduce the levels of many of

their target mRNAs. Wang *et al.* 2004 (Genome Biology 5:R65) have developed and applied a computational algorithm to predict 95 *Arabidopsis thaliana* miRNAs, which included 12 known ones and 83 new miRNAs. The 83 new miRNAs were found to be conserved with more than 90% sequence identity between the *Arabidopsis* and rice genomes. Using the Smith-Waterman nucleotide-alignment algorithm to predict mRNA targets for the 83 new miRNAs and by focusing on target sites that were conserved in both *Arabidopsis* and rice, Wang *et al.* 2004 (Genome Biology 5:R65) predicted 371 mRNA targets with an average of 4.8 targets per miRNA. A large proportion of these mRNA targets encoded proteins with transcription regulatory activity. Brennecke *et al.* 2005 (Brennecke *et al.* 2005 PLoS Biology 3: e85) have systematically evaluated the minimal requirements for functional miRNA:mRNA target duplexes *in vivo* and have grouped the target sites into two categories. The so-called 5' dominant sites have sufficient complementarity to the 5'-end on the miRNA, so that little or no pairing with the 3'-end of the miRNA is needed. The second class comprises the so-called 3' compensatory sites, which have insufficient 5'-end pairing and require strong 3'-end duplex formation in order to be functional. In addition to presenting experimental examples from both types of miRNA:target pairing *in vivo*, Brennecke *et al.* 2005 (Brennecke *et al.* 2005 PLoS Biology 3: e85) provide evidence that a given miRNA has in average ca. 100 mRNA target sites, further supporting the notion that miRNAs can regulate the expression of a large fraction of the protein-coding genes in multicellular eukaryotes.

20 *MicroRNAs and human disease*

Analysis of the genomic location of miRNAs indicates that they play important roles in human development and disease. Several human diseases have already been pinpointed in which miRNAs or their processing machinery might be implicated. One of them is spinal muscular atrophy (SMA), a paediatric neurodegenerative disease caused by reduced protein levels or loss-of-function mutations of the survival of motor neurons (SMN) gene (Paushkin *et al.* 2002, Curr.Opin.Cell Biol. 14: 305-312). Two proteins (Gemin3 and Gemin4) that are part of the SMN complex are also components of miRNPs, whereas it remains to be seen whether miRNA biogenesis or function is dysregulated in SMA and what effect this has on pathogenesis. Another neurological disease linked to mi/siRNAs is fragile X mental retardation (FXMR) caused by absence or mutations of the fragile X mental retardation protein (FMRP)(Nelson *et al.* 2003, TIBS 28: 534-540), and there are additional clues that miRNAs might play a role in other neurological diseases. Yet another interesting finding is that the miR-224 gene locus lies within the minimal candidate region of two different neurological diseases: early-onset Parkinsonism and X-linked mental retardation (Dostie *et al.* 2003, RNA: 9: 180-186). Links between cancer and miRNAs have also been recently described. The most frequent single genetic abnormality in chronic lymphocytic leukaemia (CLL) is a deletion localized to chromosome 13q14 (50% of the cases). A recent study determined that two

different miRNA (miR15 and miR16) genes are clustered and located within the intron of LEU2, which lies within the deleted minimal region of the B-cell chronic lymphocytic leukaemia (B-CLL) tumour suppressor locus, and both genes are deleted or down-regulated in the majority of CLL cases (Calin *et al.* 2002, Proc. Natl. Acad. Sci.U.S.A. 99: 15524-15529). Calin *et al.* 2004 (Calin *et al.* 2004, Proc. Natl. Acad. Sci.U.S.A. 101: 2999-3004) have further investigated the possible involvement of microRNAs in human cancers on a genome-wide basis, by mapping 186 miRNA genes and compared their location to the location of previous reported non-random genetic alterations. Interestingly, they showed that microRNA genes are frequently located at fragile sites, as well as in minimal regions of loss of heterozygosity, minimal regions of amplification (minimal amplicons), or common breakpoint regions. Overall, 98 of 186 (52.5%) of the microRNA genes in their study were in cancer-associated genomic regions or in fragile sites. Moreover, by Northern blotting, Calin *et al.* 2004 (Calin *et al.* 2004, Proc. Natl. Acad. Sci.U.S.A. 101: 2999-3004) showed that several miRNAs located in deleted regions had low levels of expression in cancer samples. These data provide the first catalog of miRNA genes that may have roles in cancer and indicate that the full complement of human miRNAs may be extensively involved in different cancers.

In a recent study, Eis *et al.* (Eis *et al.* 2005, Proc. Natl. Acad. Sci.U.S.A. 102: 3627-3632) showed that the human miR-155 is processed from sequences present in BIC RNA, which is a spliced and polyadenylated non-protein-coding RNA that accumulates in lymphoma cells. The precursor of miR-155 is most likely a transient spliced or unspliced nuclear BIC transcript rather than accumulated BIC RNA, which is primarily cytoplasmic. Eis *et al.* (Eis *et al.* 2005, Proc. Natl. Acad. Sci.U.S.A. 102: 3627-3632) also observed that clinical isolates of several types of B cell lymphomas, including diffuse large B cell lymphoma (DLBCL), have 10- to 30-fold higher copy numbers of miR-155 than do normal circulating B cells. Significantly higher levels of miR-155 were present in DLBCLs with an activated B cell phenotype than with the germinal center phenotype. Because patients with activated B cell-type DLBCL have a poorer clinical prognosis, Eis *et al.* (Eis *et al.* 2005, Proc. Natl. Acad. Sci.U.S.A. 102: 3627-3632) propose that quantification of this microRNA would be diagnostically useful.

In another recent paper, Poy *et al.* (Poy *et al.* 2004, Nature 432: 226-230) identified a novel, evolutionarily conserved and pancreatic islet-specific miRNA (miR-375), and showed that overexpression of miR-375 suppressed glucose-induced insulin secretion, and conversely, inhibition of endogenous miR-375 function enhanced insulin secretion. The mechanism by which secretion is modified by miR-375 is independent of changes in glucose metabolism or intracellular  $\text{Ca}^{2+}$ -signalling but correlated with a direct effect on insulin exocytosis. In the study, Myotrophin was validated as a target of miR-375. Inhibition of Myotrophin by small interfering (si)RNA mimicked the effects of miR-375 on glucose-stimulated insulin secretion and exocytosis. Poy *et al.* (Poy *et al.* 2004, Nature 432: 226-230) thus conclude that miR-

375 is a regulator of insulin secretion and could constitute a novel pharmacological target for the treatment of diabetes.

Yet another recent publication by Johnson *et al.* (Johnson *et al.* 2005, *Cell* 120: 635-647) showed that the let-7 miRNA family negatively regulates RAS in two different *c. elegans* 5 tissues and two different human cell lines. Another interesting finding was that let-7 is expressed in normal adult lung tissue but is poorly expressed in lung cancer cell lines and lung cancer tissue. Furthermore, the expression of let-7 inversely correlates with expression of RAS protein in lung cancer tissues, suggesting a possible causal relationship. Overexpression of let-7 inhibited growth of a lung cancer cell line in vitro, suggesting a causal 10 relationship between let-7 and cell growth in these cells. The combined results of Johnson *et al.* (Johnson *et al.* 2005, *Cell* 120: 635-647) that let-7 expression is reduced in lung tumors, that several let-7 genes map to genomic regions that are often deleted in lung cancer patients, that overexpression of let-7 can inhibit lung tumor cell line growth, that the 15 expression of the RAS oncogene is regulated by let-7, and that RAS is significantly overexpressed in lung tumor samples strongly implicate let-7 as a tumor suppressor in lung tissue and also suggests a possible mechanism.

In conclusion, it has been anticipated that connections between miRNAs and human diseases will only strengthen in parallel with the knowledge of miRNAs and the gene networks that they control. Moreover, the understanding of the regulation of RNA-mediated gene 20 expression is leading to the development of novel therapeutic approaches that will be likely to revolutionize the practice of medicine (Nelson *et al.* 2003, *TIBS* 28: 534-540).

#### *Small interfering RNAs and RNAi*

Some of the recent attention paid to small RNAs in the size range of 18 to 25 nt is due to the phenomenon RNA interference (RNAi), in which double-stranded RNA leads to the 25 degradation of any RNA that is homologous (Fire *et al.* 1998, *Nature* 391: 806-811). RNAi relies on a complex and ancient cellular mechanism that has probably evolved for protection against viral attack and mobile genetic elements. A crucial step in the RNAi mechanism is the generation of short interfering RNAs (siRNAs), double-stranded RNAs that are about 22 nt long each. The siRNAs lead to the degradation of homologous target RNA and the production 30 of more siRNAs against the same target RNA (Lipardi *et al.* 2001, *Cell* 107: 297-307). The present view for the mRNA degradation pathway of RNAi is that antiparallel Dicer dimers cleave long double-stranded dsRNAs to form siRNAs in an ATP-dependent manner. The siRNAs are then incorporated in the RNA-induced silencing complex (RISC) and ATP-dependent unwinding of the siRNAs activates RISC (Zhang *et al.* 2002, *EMBO J.* 21: 5875-

5885; Nykanen *et al.* 2001, Cell 107: 309-321). The active RISC complex is thus guided to degrade the specific target mRNAs.

*Detection and analysis of microRNAs and siRNAs*

The current view that miRNAs may represent a newly discovered, hidden layer of gene regulation has resulted in high interest among researchers around the world in the discovery of miRNAs, their targets and mechanism of action. Detection and analysis of these small RNAs is, however not trivial. Thus, the discovery of more than 1400 miRNAs to date has required taking advantage of their special features. First, the research groups have used the small size of the miRNAs as a primary criterion for isolation and detection. Consequently, 5 standard cDNA libraries would lack miRNAs, primarily because RNAs that small are normally excluded by size selection in the cDNA library construction procedure. Total RNA from fly embryos, worms or HeLa cells have been size fractionated so that only molecules 10 25 nucleotides or smaller would be captured (Moss 2002, Curr.Biology 12: R138-R140). Synthetic oligomers have then been ligated directly to the RNA pools using T4 RNA ligase. 15 Then the sequences have been reverse-transcribed, amplified by PCR, cloned and sequenced (Moss 2002, Curr.Biology 12: R138-R140). The genome databases have subsequently been queried with the sequences, confirming the origin of the miRNAs from these organisms as well as placing the miRNA genes physically in the context of other genes in the genome. The vast majority of the cloned sequences have been located in intronic regions or between 20 genes, occasionally in clusters, suggesting that the tandemly arranged miRNAs are processed from a single transcript to allow coordinate regulation. Furthermore, the genomic sequences have revealed the fold-back structures of the miRNA precursors (Moss 2002, Curr.Biology 12: R138-R140).

The size and often low level of expression of different miRNAs require the use of sensitive 25 and quantitative analysis tools. Due to their small size of 18-25 nt, the use of conventional quantitative real-time PCR for monitoring expression of mature miRNAs is excluded. Therefore, most miRNA researchers currently use Northern blot analysis combined with polyacrylamide gels to examine expression of both the mature and pre-miRNAs (Reinhart *et al.* 2000, Nature 403: 901-906; Lagos-Quintana *et al.* 2001, Science 294: 853-858; Lee and 30 Ambros 2001, Science 294: 862-864). Primer extension has also been used to detect the mature miRNA (Zeng and Cullen 2003, RNA 9: 112-123). The disadvantage of all the gel-based assays (Northern blotting, primer extension, RNase protection assays etc.) as tools for monitoring miRNA expression includes low throughput and poor sensitivity. Consequently, a large amount of total RNA per sample is required for Northern analysis of miRNAs, which is 35 not feasible when the cell or tissue source is limited.

DNA microarrays would appear to be a good alternative to Northern blot analysis to quantify miRNAs in a genome-wide scale, since microarrays have excellent throughput. Krichevsky *et al.* 2003 used cDNA microarrays to monitor the expression of miRNAs during neuronal development with 5 to 10 µg aliquot of input total RNA as target, but the mature miRNAs had 5 to be separated from the miRNA precursors using micro concentrators prior to microarray hybridizations (Krichevsky *et al.* 2003, RNA 9: 1274-1281). Liu *et al* 2004 (Liu *et al.* 2004, Proc.Natl. Acad. Sci. U.S.A 101:9740-9744) have developed a microarray for expression profiling of 245 human and mouse miRNAs using 40-mer DNA oligonucleotide capture probes. Thomson *et al.* 2004 (Thomson *et al.* 2004, Nature Methods 1: 1-6) describe the 10 development of a custom oligonucleotide microarray platform for expression profiling of 124 mammalian miRNAs conserved in human and mouse using oligonucleotide capture probes complementary to the mature microRNAs. The microarray was used in expression profiling of the 124 miRNAs in question in different adult mouse tissues and embryonic stages. A similar approach was used by Miska *et al.* 2004 (Genome Biology 2004; 5:R68) for the development 15 of an oligoarray for expression profiling of 138 mammalian miRNAs, including 68 miRNAs from rat and monkey brains. Yet another approach was taken by Barad *et al.* 2004 (Genome Research 2004; 14: 2486-2494), who developed a 60-mer oligonucleotide microarray platform for known human mature miRNAs and their precursors. The drawback of all DNA-based oligonucleotide arrays regardless of the capture probe length is the requirement of 20 high concentrations of labelled input target RNA for efficient hybridization and signal generation, low sensitivity for rare and low-abundant miRNAs, and the necessity for post-array validation using more sensitive assays such as real-time quantitative PCR, which is not currently feasible. In addition, at least in some array platforms discrimination of highly homologous miRNA differing by just one or two nucleotides could not be achieved, thus 25 presenting problems in data interpretation, although the 60-mer microarray by Barad *et al.* 2004 (Genome Research 2004; 14: 2486-2494) appears to have adequate specificity.

A PCR approach has also been used to determine the expression levels of mature miRNAs (Grad *et al.* 2003, Mol. Cell 11: 1253-1263). This method is useful to clone miRNAs, but highly impractical for routine miRNA expression profiling, since it involves gel isolation of 30 small RNAs and ligation to linker oligonucleotides. Allawi *et al.* (2004, RNA 10: 1153-1161) have developed a method for quantitation of mature miRNAs using a modified Invader assay. Although apparently sensitive and specific for the mature miRNA, the drawback of the Invader quantitation assay is the number of oligonucleotide probes and individual reaction steps needed for the complete assay, which increases the risk of cross-contamination 35 between different assays and samples, especially when high-throughput analyses are desired. Schmittgen *et al.* (2004, Nucleic Acids Res. 32: e43) describe an alternative method to Northern blot analysis, in which they use real-time PCR assays to quantify the expression of miRNA precursors. The disadvantage of this method is that it only allows quantification of

the precursor miRNAs, which does not necessarily reflect the expression levels of mature miRNAs. In order to fully characterize the expression of large numbers of miRNAs, it is necessary to quantify the mature miRNAs, such as those expressed in human disease, where alterations in miRNA biogenesis produce levels of mature miRNAs that are very different from 5 those of the precursor miRNA. For example, the precursors of 26 miRNAs were equally expressed in non-cancerous and cancerous colorectal tissues from patients, whereas the expression of mature human miR143 and miR145 was greatly reduced in cancer tissues compared with non-cancer tissues, suggesting altered processing for specific miRNAs in human disease (Michael *et al.* 2003, Mol. Cancer Res. 1: 882-891). On the other hand, recent 10 findings in maize with miR166 and miR165 in *Arabidopsis thaliana*, indicate that microRNAs act as signals to specify leaf polarity in plants and may even form movable signals that emanate from a signalling centre below the incipient leaf (Juarez *et al.* 2004, Nature 428: 84-88; Kidner and Martienssen 2004, Nature 428: 81-84).

Most of the miRNA expression studies in animals and plants have utilized Northern blot 15 analysis, tissue-specific small RNA cloning and expression profiling by microarrays or real-time PCR of the miRNA hairpin precursors, as described above. However, these techniques lack the resolution for addressing the spatial and temporal expression patterns of mature miRNAs. Due to the small size of mature miRNAs, detection of them by standard RNA *in situ* hybridization has proven difficult to adapt in both plants and vertebrates, even though *in situ* 20 hybridization has recently been reported in *A. thaliana* and maize using RNA probes corresponding to the stem-loop precursor miRNAs (Chen *et al.* 2004, Science 203: 2022-2025; Juarez *et al.* 2004, Nature 428: 84-88). Brennecke *et al.* 2003 (Cell 113: 25-36) and Mansfield *et al.* 2004 (Nature Genetics 36: 1079-83) report on an alternative method in 25 which reporter transgenes, so-called sensors, are designed and generated to detect the presence of a given miRNA in an embryo. Each sensor contains a constitutively expressed reporter gene (e.g. lacZ or green fluorescent protein) harbouring miRNA target sites in its 3'-UTR. Thus, in cells that lack the miRNA in question, the transgene RNA is stable allowing detection of the reporter, whereas cells expressing the miRNA, the sensor mRNA is targeted 30 for degradation by the RNAi pathway. Although sensitive, this approach is time-consuming since it requires generation of the expression constructs and transgenes. Furthermore, the sensor-based technique detects the spatiotemporal miRNA expression patterns via an indirect method as opposed to direct *in situ* hybridization of the mature miRNAs.

The large number of miRNAs along with their small size makes it difficult to create loss-of-function mutants for functional genomics analyses. Another potential problem is that many 35 miRNA genes are present in several copies per genome occurring in different loci, which makes it even more difficult to obtain mutant phenotypes. Boutla *et al.* 2003 (Nucleic Acids Research 31: 4973-4980) describe the use of DNA antisense oligonucleotides complementary

to 11 different miRNAs in *Drosophila* as well as their use to inactivate the miRNAs by injecting the DNA oligonucleotides into fly embryos. Of the 11 DNA antisense oligonucleotides, only 4 constructs showed severe interference with normal development, while the remaining 7 oligonucleotides didn't show any phenotypes presumably due to their inability to inhibit the 5 miRNA in question. Thus, the success rate for using DNA antisense oligonucleotides to inhibit miRNA function would most likely be too low to allow functional analyses of miRNAs on a larger, genomic scale. An alternative approach to this has been reported by Hutvagner et al. 2004 (PLoS Biology 2: 1-11), in which 2'-O-methyl antisense oligonucleotides could be used 10 as potent and irreversible inhibitors of siRNA and miRNA function *in vitro* and *in vivo* in *Drosophila* and *c. elegans*, thereby inducing a loss-of-function phenotype. A drawback of this method is the need of high 2'-O-methyl oligonucleotide concentrations (100 micromolar) in transfection and injection experiments, which may be toxic to the animal.

In conclusion, the biggest challenge in detection, quantitation and functional analysis of the mature miRNAs as well as siRNAs using currently available methods is their small size of the 15 of 18-25 nt and often low level of expression. The present invention provides the design and development of novel oligonucleotide compositions and probe sequences for accurate, highly sensitive and specific detection and functional analysis of miRNAs, their target mRNAs and siRNA transcripts.

#### *RNA editing and alternative splicing*

20 RNA editing is used to describe any specific change in the primary sequence of an RNA molecule, excluding other mechanistically defined processes such as alternative splicing or polyadenylation. RNA alterations due to editing fall into two broad categories, depending on whether the change happens at the base or nucleotide level (Gott 2003, C. R. Biologies 326 901-908). RNA editing is quite widespread, occurring in mammals, viruses, marsupials, 25 plants, flies, frogs, worms, squid, fungi, slime molds, dinoflagellates, kinetoplastid protozoa, and other unicellular eukaryotes. The current list most likely represents only the tip of the iceberg; based on the distribution of homologues of known editing enzymes, as RNA editing almost certainly occurs in many other species, including all metazoa. Since RNA editing can be regulated in a developmental or tissue-specific manner, it is likely to play a significant role 30 in the etiology of human disease (Gott 2003, C. R. Biologies 326 901-908).

A common feature for eukaryotic genes is that they are composed of protein-encoding exons and introns. Introns are characterized by being excised from the pre-mRNA molecule in RNA splicing, as the sequences on each side of the intron are spliced together. RNA splicing not only provides functional mRNA, but is also responsible for generating additional diversity. 35 This phenomenon is called alternative splicing, which results in the production of different

mRNAs from the same gene. The mRNAs that represent isoforms arising from a single gene can differ by the use of alternative exons or retention of an intron that disrupts two exons. This process often leads to different protein products that may have related or drastically different, even antagonistic, cellular functions. There is increasing evidence indicating that 5 alternative splicing is very widespread (Croft *et al.* Nature Genetics, 2000). Recent studies have revealed that at least 80% of the roughly 35,000 genes in the human genome are alternatively spliced (Kampa *et al.* 2004, Genome Research 14: 331-342). Clearly, by combining different types of modifications and thus generating different possible combinations of transcripts of different genes, alternative splicing together with RNA editing 10 are potent mechanisms for generating protein diversity. Analysis of the alternative splice variants and RNA editing, in turn, represents a novel approach to functional genomics, disease diagnostics and pharmacogenomics.

*Misplaced control of alternative splicing as a causative agent for human disease*

The detection of the detailed structure of the transcriptional output is an important goal for 15 molecular characterization of a cell or tissue. Without the ability to detect and quantify the splice variants present in one tissue, the transcript content or the protein content cannot be described accurately. Molecular medical research shows that many cancers result in altered levels of splice variants, so an accurate method to detect and quantify these transcripts is required. Mutations that produce an aberrant splice form can also be the primary cause of 20 such severe diseases such as spinal muscular dystrophy and cystic fibrosis.

Much of the study of human disease, indeed much of genetics is based upon the study of a few model organisms. The evolutionary stability of alternative splicing patterns and the degree to which splicing changes according to mutations and environmental and cellular conditions influence the relevance of these model systems. At present, there is little 25 understanding of the rates at which alternative splicing patterns or RNA editing change, and the factors influencing these rates.

Previously, other analysis methods have been performed with the aim of detecting either splicing of RNA transcripts *per se* in yeast, or of detecting putative exon skipping splicing events in rat tissues, but neither of these approaches had sufficient resolution to estimate 30 quantities of splice variants, a factor that could be essential to an understanding of the changes in cell life cycle and disease. Thus, improved methods are needed for nucleic acid hybridization and quantitation. The present method of invention enables discrimination between mRNA splice variants as well as RNA-edited transcripts and detects each variant in a nucleic acid sample, such as a sample derived from a patient in e.g. addressing the 35 spatiotemporal expression patterns by RNA *in situ* hybridization.

*Antisense transcription in eukaryotes*

RNA-mediated gene regulation is widespread in higher eukaryotes and complex genetic phenomena like RNA interference, co-suppression, transgene silencing, imprinting, methylation, and possibly position-effect variegation and transvection, all involve intersecting pathways based on or connected to RNA signalling (Mattick 2001; EMBO reports 2, 11: 986-991). Recent studies indicate that antisense transcription is a very common phenomenon in the mouse and human genomes (Okazaki *et al.* 2002; Nature 420: 563-573; Yelin *et al.* 2003, Nature Biotechnol.). Thus, antisense modulation of gene expression in eukaryotic cells, e.g. human cells appear to be a common regulatory mechanism. In light of this, the present invention provides a method for detection and functional analysis of non-coding antisense RNAs, as well as a method for detecting the overlapping regions between sense-antisense transcriptional units.

*Cancer diagnosis and identification of tumor origin*

Cancer classification relies on the subjective interpretation of both clinical and histopathological information by eye with the aim of classifying tumors in generally accepted categories based on the tissue of origin of the tumor. However, clinical information can be incomplete or misleading. In addition, there is a wide spectrum in cancer morphology and many tumors are atypical or lack morphologic features that are useful for differential diagnosis. These difficulties may result in diagnostic confusion, with the need for mandatory second opinions in all surgical pathology cases (Tomaszewski and LiVolsi 1999, Cancer 86: 2198-2200).

Molecular diagnostics offer the promise of precise, objective, and systematic human cancer classification, but these tests are not widely applied because characteristic molecular markers for most solid tumors have yet to be identified. In the recent years microarray-based tumor gene expression profiling has been used for cancer diagnosis. However, studies are still limited and have utilized different array platforms making it difficult to compare the different datasets (Golub *et al.* 1999, Science 286: 531-537; Alizadeh *et al.* 2000, Nature 403: 503-511; Bittner *et al.* 2000, Nature 406: 536-540). In addition, comprehensive gene expression databases have to be developed, and there are no established analytical methods yet capable of solving complex, multiclass, gene expression-based classification problems.

Another problem for cancer diagnostics is the identification of tumor origin for metastatic carcinomas. For example, in the United States, 51,000 patients (4% of all new cancer

cases) present annually with metastases arising from occult primary carcinomas of unknown origin (ACS Cancer Facts & Figures 2001: American Cancer Society). Adenocarcinomas represent the most common metastatic tumors of unknown primary site. Although these patients often present at a late stage, the outcome can be positively affected by accurate 5 diagnoses followed by appropriate therapeutic regimens specific to different types of adenocarcinoma (Hillen 2000, Postgrad. Med. 3. 76: 690-693). The lack of unique microscopic appearance of the different types of adenocarcinomas challenges morphological diagnosis of adenocarcinomas of unknown origin. The application of tumor-specific serum markers in identifying cancer type could be feasible, but such markers are not available at 10 present (Milovic *et al.* 2002, Med. Sci. Monit. 8: MT25-MT30). Microarray expression profiling has recently been used to successfully classify tumors according to their site of origin (Ramaswamy *et al.* 2001, Proc. Natl. Acad. Sci. U.S.A. 98: 15149-15154), but the lack of a standard for array data collection and analysis make them difficult to use in a clinical setting. SAGE (serial analysis of gene expression), on the other hand, measures absolute expression 15 levels through a tag counting approach, allowing data to be obtained and compared from different samples. The drawback of this method is, however, its low throughput, making it inappropriate for routine clinical applications. Quantitative real-time PCR is a reliable method for assessing gene expression levels from relatively small amounts of tissue (Bustin 2002, J. Mol. Endocrinol. 29: 23-39). Although this approach has recently been successfully applied to 20 the molecular classification of breast tumors into prognostic subgroups based on the analysis of 2,400 genes (Iwao *et al.* 2002, Hum. Mol. Genet. 11: 199-206), the measurement of such a large number of randomly selected genes by PCR is clinically impractical.

Since the discovery of the first miRNA gene lin-4, in 1993, microRNAs have emerged as important non-coding RNAs, involved in a wide variety of regulatory functions during cell 25 growth, development and differentiation. Furthermore, an expanding inventory of microRNA studies has shown that many miRNAs are mutated or down-regulated in human cancers, implying that miRNAs can act as tumor suppressors or even oncogenes. Thus, detection and quantitation of all the microRNAs with a role in human disease, including cancers, would be highly useful as biomarkers for diagnostic purposes or as novel pharmacological targets for 30 treatment. The biggest challenge, on the other hand, in detection and quantitation of the mature miRNAs using currently available methods is the small size of 18-25 nt and sometimes low level of expression.

The present invention solves the abovementioned problems by providing the design and development of novel oligonucleotide compositions and probe sequences for accurate, highly 35 sensitive and specific detection and quantitation of microRNAs and other non-coding RNAs, useful as biomarkers for diagnostic purposes of human disease as well as for antisense-based intervention, which is targeted against tumorigenic miRNAs and other non-coding RNAs. The

invention furthermore provides novel oligonucleotide compositions and probe sequences for sensitive and specific detection and quantitation of microRNAs, useful as biomarkers for the identification of the primary site of metastatic tumors of unknown origin.

#### SUMMARY OF THE INVENTION

5 The challenges of establishing genome function and understanding the layers of information hidden in the complex transcriptomes of higher eukaryotes call for novel, improved technologies for detection and analysis of non-coding RNA and protein-coding RNA molecules in complex nucleic acid samples. Thus, it would be highly desirable to be able to detect and analyse the expression of mature microRNAs, siRNAs, RNA-edited transcripts as well as  
10 highly homologous splice variants in the transcriptomes of eukaryotes using methods based on specific and sensitive oligonucleotide detection probes.

The present invention solves the current problems faced by conventional approaches used in detection and analysis of mature miRNAs, their target mRNAs as well as siRNAs as outlined above by providing a method for the design, synthesis and use of novel oligonucleotide  
15 compositions and probe sequences with improved sensitivity and high sequence specificity for RNA target sequences, such as mature miRNAs and siRNAs- so that they are unlikely to detect a random RNA target molecule. Such oligonucleotide probes comprise a recognition sequence complementary to the RNA target sequence, which said recognition sequence is substituted with high-affinity nucleotide analogues, e.g. LNA, to increase the sensitivity and  
20 specificity of conventional oligonucleotides, such as DNA oligonucleotides, for hybridization to short target sequences, e.g. mature miRNAs, stem-loop precursor miRNAs, siRNAs or other non-coding RNAs as well as miRNA binding sites in their cognate mRNA targets, mRNAs, mRNA splice variants, RNA-edited mRNAs and antisense RNAs. The invention features a method of designing the detection probe sequences by selecting optimal substitution patterns  
25 for the high-affinity analogues, e.g. LNAs for the detection probes. This method involves (a) substituting the detection probe sequence with the high affinity analogue LNA in chimeric LNA-DNA oligonucleotides using regular spacing between the LNA substitutions, e.g. at every second nucleotide position, every third nucleotide position, or every fourth nucleotide position, in order to promote the A-type duplex geometry between the substituted detection  
30 probe and its complementary RNA target; with the said LNA monomer substitutions spiked in all the possible phases in the probe sequence with an unsubstituted monomer at the 5'-end position and 3'-end position in all the substituted designs; (b) determining the ability of the designed detection probes with different regular substitution patterns to self-anneal; and (c) determining the melting temperature of the substituted probes sequences of the invention,

and (d) selecting the probe sequences with the highest melting temperatures and lowest self-complementarity score, i.e. lowest ability to self-anneal are selected.

Another aspect the invention features a method of designing the detection probe sequences by selecting optimal substitution patterns for the LNAs, which said method involves  
5 substituting the detection probe sequence with the high affinity analogue LIMA in chimeric LNA-DNA oligonucleotides using irregular spacing between the LNA monomers and selecting the probe sequences with the highest melting temperatures and lowest self-complementarity score. In yet another aspect the invention features a computer code for a preferred software program of the invention for the design and selection of the said substituted detection probe  
10 sequences.

The present invention hence also relates to a collection of detection probes, wherein each member of said collection comprises a recognition sequence consisting of nucleobases and affinity enhancing nucleobase analogues, and wherein the recognition sequences exhibit a combination of high melting temperatures and low self-complementarity scores, said melting  
15 temperatures being the melting temperature of the duplex between the recognition sequence and its complementary DNA or RNA sequence.

Also single probes taken from such a collection form part of the present invention.

The invention also relates to a method for A method for expanding or building a collection defined above, comprising  
20 A) defining a reference nucleotide sequence consisting of nucleobases, said reference nucleotide sequence being complementary to a target sequence for which the collection does not contain a detection probe,  
B) substituting the reference nucleotide sequence's nucleobases with affinity enhancing nucleobase analogues to provide a set of chimeric sequences wherein,  
25 C) determining usefulness of each of the chimeric sequences based on assessment of their ability to self-anneal and their melting temperature, and  
D) synthesizing and adding, to the collection, a probe comprising as its recognition sequence the chimeric sequence with the optimum combination of high melting temperature and low self-annealing.  
30 Also part of the invention is a method for designing an optimized detection probe for a target nucleotide sequence, comprising  
1) defining a reference nucleotide sequence consisting of nucleobases, said reference nucleotide sequence being complementary to said target nucleotide sequence,  
2) substituting the reference nucleotide sequence's nucleobases with affinity enhancing

nucleobase analogues to provide a set of chimeric sequences

3) determining usefulness of each of the chimeric sequences based on assessment of their ability to self-anneal and their melting temperatures, and

4) defining the optimized detection probe as the one in the set having as its recognition

5 sequence the chimeric sequence with the optimum combination of high melting temperature and low self-annealing.

Furthermore, the present invention also relates to a computer system for designing an optimized detection probe for a target nucleic acid sequence, said system comprising

a) input means for inputting the target nucleotide,

10 b) storage means for storing the target nucleotide sequence,

c) optionally executable code which can calculate a reference nucleotide sequence being complementary to said target nucleotide sequence and/or input means for inputting the reference nucleotide sequence,

d) optionally storage means for storing the reference nucleotide sequence,

15 e) executable code which can generate chimeric sequences from the reference nucleotide sequence or the target nucleic acid sequence, wherein said chimeric sequences comprise the reference nucleotide sequence, wherein has been in-substituted affinity enhancing nucleobase analogues,

f) executable code which can determine the usefulness of such chimeric sequences based on 20 assessment of their ability to self-anneal and their melting temperatures and either rank such chimeric sequences according to their usefulness,

g) storage means for storing at least one chimeric sequence, and

h) output means for presenting the sequence of at least one optimized detection probe.

Also a storage means embedding executable code (e.g. a computer program) which executes

25 the design steps of the method referred to above is part of the present invention.

Further, the present invention also relates to a method for specific isolation, purification,

amplification, detection, identification, quantification, inhibition or capture of a target

nucleotide sequence in a sample, said method comprising contacting said sample with a member of a collection defined above under conditions that facilitate hybridization between

30 said member/probe and said target nucleotide sequence.

In another aspect the invention features detection probe sequences containing a ligand,

which said ligand means something, which binds. Such ligand-containing detection probes of the invention are useful for isolating target RNA molecules from complex nucleic acid mixtures, such as miRNAs, their cognate target mRNAs and siRNAs. Ligands comprise biotin

35 and functional groups such as: aromatic groups (such as benzene, pyridine, naphthalene,

anthracene, and phenanthrene), heteroaromatic groups (such as thiophene, furan, tetrahydrofuran, pyridine, dioxane, and pyrimidine), carboxylic acids, carboxylic acid esters, carboxylic acid halides, carboxylic acid azides, carboxylic acid hydrazides, sulfonic acids, sulfonic acid esters, sulfonic acid halides, semicarbazides, thiosemicarbazides, aldehydes, 5 ketones, primary alcohols, secondary alcohols, tertiary alcohols, phenols, alkyl halides, thiols, disulphides, primary amines, secondary amines, tertiary amines, hydrazines, epoxides, maleimides, C1-C20 alkyl groups optionally interrupted or terminated with one or more heteroatoms such as oxygen atoms, nitrogen atoms, and/or sulphur atoms, optionally containing aromatic or mono/polyunsaturated hydrocarbons, polyoxyethylene such as 10 polyethylene glycol, oligo/polyamides such as poly- $\beta$ -alanine, polyglycine, polylysine, peptides, oligo/polysaccharides, oligo/polyphosphates, toxins, antibiotics, cell poisons, and steroids, and also affinity ligands, i.e. functional groups or biomolecules that have a specific affinity for sites on particular proteins, antibodies, poly- and oligosaccharides, and other biomolecules.

15 In another aspect the invention features detection probe sequences, which said sequences have been furthermore modified by Selectively Binding Complementary (SBC) nucleobases, i.e. modified nucleobases that can make stable hydrogen bonds to their complementary nucleobases, but are unable to make stable hydrogen bonds to other SBC nucleobases. Such SBC monomer substitutions are especially useful when highly self-complementary detection 20 probe sequences are employed. As an example, the SBC nucleobase A', can make a stable hydrogen bonded pair with its complementary unmodified nucleobase, T. Likewise, the SBC nucleobase T' can make a stable hydrogen bonded pair with its complementary unmodified nucleobase, A. However, the SBC nucleobases A' and T' will form an unstable hydrogen bonded pair as compared to the base pairs A'-T and A-T'. Likewise, a SBC nucleobase of C is 25 designated C and can make a stable hydrogen bonded pair with its complementary unmodified nucleobase G, and a SBC nucleobase of G is designated G' and can make a stable hydrogen bonded pair with its complementary unmodified nucleobase C, yet C and G' will form an unstable hydrogen bonded pair as compared to the base pairs C-G and C-G'. A stable hydrogen bonded pair is obtained when 2 or more hydrogen bonds are formed e.g. the 30 pair between A' and T, A and T', C and G', and C and G. An unstable hydrogen bonded pair is obtained when 1 or no hydrogen bonds is formed e.g. the pair between A' and T', and C and G'. Especially interesting SBC nucleobases are 2,6-diaminopurine (A', also called D) together with 2-thio-uracil (U', also called 2SU)(2-thio-4-oxo-pyrimidine) and 2-thio-thymine (T', also called 2ST)(2-thio-4-oxo-5-methyl-pyrimidine).

35 In another aspect the detection probe sequences of the invention are covalently bonded to a solid support by reaction of a nucleoside phosphoramidite with an activated solid support, and subsequent reaction of a nucleoside phosphoramido with an activated nucleotide or

nucleic acid bound to the solid support. In some embodiments, the solid support or the detection probe sequences bound to the solid support are activated by illumination, a photogenerated acid, or electric current. In other embodiments the detection probe sequences contain a spacer, e.g. a randomized nucleotide sequence or a non-base sequence, 5 such as hexaethylene glycol, between the reactive group and the recognition sequence. Such covalently bonded detection probe sequence populations are highly useful for large-scale detection and expression profiling of mature miRNAs, stem-loop precursor miRNAs, siRNAs and other non-coding RNAs.

The present oligonucleotide compositions and detection probe sequences of the invention are 10 highly useful and applicable for detection of individual small RNA molecules in complex mixtures composed of hundreds of thousands of different nucleic acids, such as detecting mature miRNAs, their target mRNAs or siRNAs, by Northern blot analysis or for addressing the spatiotemporal expression patterns of miRNAs, siRNAs or other non-coding RNAs as well as mRNAs by *in situ* hybridization in whole-mount embryos, whole-mount animals or plants 15 or tissue sections of plants or animals, such as human, mouse, rat, zebrafish, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, rice and maize. The present oligonucleotide compositions and detection probe sequences of invention are furthermore highly useful and applicable for large-scale and genome-wide expression profiling of mature 20 miRNAs, siRNAs or other non-coding RNAs in animals and plants by oligonucleotide microarrays. The present oligonucleotide compositions and detection probe sequences are furthermore highly useful in functional analysis of miRNAs, siRNAs or other non-coding RNAs *in vitro* and *in vivo* in plants or animals, such as human, mouse, rat, zebrafish, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, rice and maize, by 25 inhibiting their mode of action, e.g. the binding of mature miRNAs to their cognate target mRNAs. The oligonucleotide compositions and detection probe sequences of invention are also applicable to detecting, testing, diagnosing or quantifying miRNAs, siRNAs, other non-coding RNAs, RNA-edited transcripts or alternative mRNA splice variants implicated in or connected to human disease in complex human nucleic acid samples, e.g. from cancer patients. The oligonucleotide compositions and probe sequences are especially applicable for 30 accurate, highly sensitive and specific detection and quantitation of microRNAs and other non-coding RNAs, which are useful as biomarkers for diagnostic purposes of human diseases, such as cancers, as well as for antisense-based intervention, targeted against tumorigenic miRNAs and other non-coding RNAs. The novel oligonucleotide compositions and probe sequences are furthermore applicable for sensitive and specific detection and quantitation of 35 microRNAs, which can be used as biomarkers for the identification of the primary site of metastatic tumors of unknown origin.

Brief Description Of The Drawings

Fig. 1: The structures of DNA, LNA and RNA nucleosides.

Fig. 2: The structures of LNA 2,6-diaminopurine and LNA 2-thiothymidine nucleosides.

Fig.: 3. The specificity of microRNA detection by *in situ* hybridization with LNA-substituted 5 probes.

The LNA probes containing one (1 MM) or two (2 MM) mismatches were designed for the three different miRNAs miR-206, miR-124a and miR-122a (see Table 3 below). The hybridizations were performed on embryos at 72 hours post fertilization at the same temperature as the perfect match probe (0 MM).

10 Fig. 4: Examples of miRNA whole-mount *in situ* expression patterns in zebrafish detected by LNA-substituted probes.

Representatives for miRNAs expressed in the organ systems are shown. miRNAs were expressed in: (A) liver of the digestive system, (B) brain, spinal cord and cranial nerves/ganglia of the central and peripheral nervous systems, (C, M) muscles, (D) restricted 15 parts along the head-to-tail axis, (E) pigment cells of the skin, (F, L) pronephros and presumably mucous cells of the excretory system, (G, M) cartilage of the skeletal system, (H) thymus, (I, N) blood vessels of the circulatory system, (J) lateral line system of the sensory organs. Embryos in (K, L, M, N) are higher magnifications of the embryos in (C, D, G, I), respectively. (A-J, N) are lateral views; (K-M) are dorsal views. All embryos are 72 hours 20 post fertilization, except for (H), which is a five-day old larva.

Fig. 5: Detection of let-7a miRNA by *in situ* hybridization in paraffin-embedded mouse brain sections using 3' digoxigenin-labeled LNA probe.

Part of the hippocampus can be seen as an arrow-like structure.

25 Fig. 6: Detection of let-7a miRNA by *in situ* hybridization in paraffin-embedded mouse brain sections using 3' digoxigenin-labeled LNA probe.

The Purkinje cells can be seen in the cerebellum.

Fig. 7: Detection of miR-124a, miR-122a and miR-206 with DIG-labeled DNA and LNA probes in 72h zebrafish embryos.

30 (a) Dot-blot of DIG labeled DNA and LNA probes. Per probe, 1 pmol was spotted on a positively charged nylon membrane. All probes show approximately equal incorporation of the DIG-label.

(b) Only LNA probes give clear staining. LNA probes were hybridized at 59 °C (miR-122a and miR-124a) and 54 °C (miR-206). DNA probes were hybridized at 45°C.

Fig. 8 : Determination of the optimal hybridization temperature and time for in situ hybridization on 72h zebrafish embryos using LNA probes.

5 (a) LNA probes for miR-122a and miR-206 were hybridized at different temperatures. The optimal hybridization temperature lies around 21 °C below the calculated Tm of the probe. While specific staining remains at the lower temperatures, background increases significantly. At higher temperatures staining is completely lost.

10 (b) Hybridization time series with probes for miR-122a and miR-206. An incubation time of 10 min is already sufficient to get a detectable signal, while increasing the hybridization time beyond one hour does not increase the signal significantly. All in situ hybridizations were performed in parallel.

Fig. 9 : Assessment of the specificity of LNA probes using perfectly matched and mismatched probes for the detection of miR-124a, miR-122a and miR-206 by in situ hybridization on 72h zebrafish embryos.

15 Mismatched probes were hybridized under the same conditions as the perfectly matching probe. In most cases a central single mismatch is sufficient to loose signal. For the very highly expressed miR~124a specific staining was only lost upon introduction of two consecutive central mismatches in the probe.

20 Fig. 10: In situ detection of miR-124a and miR-206 in 72h zebrafish embryos using shorter LNA probe versions.

In situ hybridizations were performed with probes of 2, 4, 6, 8, 10, 12 and 14 nt shorter than the original 22nt probes. Signals of probes that were 14 nt in length still resulted in readily detectable and specific signals. A single central mismatch in the 14 nt probes for miR-124a and miR-206 prevents hybridization. Probes that were 12 nt in length gave slightly reduced staining for both miR-124a and miR-206. Staining was virtually lost when 10 and 8 nt probes were used, although weak staining in the brain could still be observed for the highly expressed miR-124a.

25

Fig. 11: In situ hybridizations for miRNAs on *Xenopus tropicalis* and mouse embryos.

30 (a) Expression of miR-1 is restricted to the muscles in the body and the head in *X. tropicalis*. miR-124a is expressed throughout the central nervous system.

(b) Expression of 15 miRNAs in 9.5 and 10.5 dpc (days post coitum) mouse embryos: miR-10a and 10b, posterior trunk; miR-196a, tailbud; miR-126, blood vessels; miR-125b, midbrain hindbrain boundary; miR-219, midbrain, hindbrain and spinal cord; miR-124a, central nervous system; miR-9, forebrain and the spinal cord; miR-206, somites; miR-1,

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heart and somites; miR-182, miR-96 and miR-183, cranial and dorsal root ganglia; miR-17-5p and miR-20 are expressed ubiquitously, like the other members of its genomic cluster.

### Definitions

For the purposes of the subsequent detailed description of the invention the following 5 definitions are provided for specific terms, which are used in the disclosure of the present invention:

In the present context "ligand" means something, which binds. Ligands comprise biotin and functional groups such as: aromatic groups (such as benzene, pyridine, naphthalene, anthracene, and phenanthrene), heteroaromatic groups (such as thiophene, furan, 10 tetrahydrofuran, pyridine, dioxane, and pyrimidine), carboxylic acids, carboxylic acid esters, carboxylic acid halides, carboxylic acid azides, carboxylic acid hydrazides, sulfonic acids, sulfonic acid esters, sulfonic acid halides, semicarbazides, thiosemicarbazides, aldehydes, ketones, primary alcohols, secondary alcohols, tertiary alcohols, phenols, alkyl halides, thiols, disulphides, primary amines, secondary amines, tertiary amines, hydrazines, epoxides, 15 maleimides, Ci-C<sub>20</sub> alkyl groups optionally interrupted or terminated with one or more heteroatoms such as oxygen atoms, nitrogen atoms, and/or sulphur atoms, optionally containing aromatic or mono/polyunsaturated hydrocarbons, polyoxyethylene such as polyethylene glycol, oligo/polyamides such as poly-β-alanine, polyglycine, polylysine, peptides, oligo/polysaccharides, oligo/polyphosphates, toxins, antibiotics, cell poisons, and 20 steroids, and also "affinity ligands", *i.e.* functional groups or biomolecules that have a specific affinity for sites on particular proteins, antibodies, poly- and oligosaccharides, and other biomolecules.

The singular form "a", "an" and "the" include plural references unless the context clearly 25 dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof. The term "a nucleic acid molecule" includes a plurality of nucleic acid molecules.

"Transcriptome" refers to the complete collection of transcriptional units of the genome of any species. In addition to protein-coding mRNAs, it also represents non-coding RNAs, such as small nucleolar RNAs, siRNAs, microRNAs and antisense RNAs, which comprise important 30 structural and regulatory roles in the cell.

A "multi-probe library" or "library of multi-probes" comprises a plurality of multi- probes, such that the sum of the probes in the library are able to recognise a major proportion of a transcriptome, including the most abundant sequences, such that about 60%, about 70%,

about 80%, about 85%, more preferably about 90%, and still more preferably 95%, of the target nucleic acids in the transcriptome, are detected by the probes.

"Sample" refers to a sample of cells, or tissue or fluid isolated from an organism or organisms, including but not limited to, for example, skin, plasma, serum, spinal fluid, lymph 5 fluid, synovial fluid, urine, tears, blood cells, organs, tumours, and also to samples of *in vitro* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, recombinant cells and cell components).

An "organism" refers to a living entity, including but not limited to, for example, human, mouse, rat, *Drosophila*, *C. elegans*, yeast, *Arabidopsis thaliana*, maize, rice, zebra fish, 10 primates, domestic animals, etc.

The terms "Detection probes" or "detection probe" or "detection probe sequence" refer to an oligonucleotide, which oligonucleotide comprises a recognition sequence complementary to a RNA (or DNA) target sequence, which said recognition sequence is substituted with high-affinity nucleotide analogues, e.g. LNA, to increase the sensitivity and specificity of 15 conventional oligonucleotides, such as DNA oligonucleotides, for hybridization to short target sequences, e.g. mature miRNAs, stem-loop precursor miRNAs, pri-miRNAs, siRNAs or other non-coding RNAs as well as miRNA binding sites in their cognate mRNA targets, mRNAs, mRNA splice variants, RNA-edited mRNAs and antisense RNAs.

The terms "miRNA" and "microRNA" refer to 18-25 nt non-coding RNAs derived from 20 endogenous genes. They are processed from longer (ca 75 nt) hairpin-like precursors termed pre-miRNAs. MicroRNAs assemble in complexes termed miRNPs and recognize their targets by antisense complementarity. If the microRNAs match 100% their target, i.e. the complementarity is complete, the target mRNA is cleaved, and the miRNA acts like a siRNA. If the match is incomplete, i.e. the complementarity is partial, then the translation of the 25 target mRNA is blocked.

The terms "Small interfering RNAs" or "siRNAs" refer to 21-25 nt RNAs derived from 30 processing of linear double-stranded RNA. siRNAs assemble in complexes termed RISC (RNA-induced silencing complex) and target homologous RNA sequences for endonucleolytic cleavage. Synthetic siRNAs also recruit RISCs and are capable of cleaving homologous RNA sequences

The term "RNA interference" (RNAi) refers to a phenomenon where double-stranded RNA homologous to a target mRNA leads to degradation of the targeted mRNA. More broadly defined as degradation of target mRNAs by homologous siRNAs.

The term "Recognition sequence" refers to a nucleotide sequence that is complementary to a region within the target nucleotide sequence essential for sequence-specific hybridization between the target nucleotide sequence and the recognition sequence.

5 The term "label" as used herein refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetric, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like.

As used herein, the terms "nucleic acid", "polynucleotide" and "oligonucleotide" refer to primers, probes, oligomer fragments to be detected, oligomer controls and unlabelled 10 blocking oligomers and shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), and to any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base, or modified purine or pyrimidine bases. There is no intended distinction in length between the term "nucleic acid", "polynucleotide" and "oligonucleotide", and these terms will be used interchangeably. These 15 terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single stranded RNA. The oligonucleotide is comprised of a sequence of approximately at least 3 nucleotides, preferably at least about 6 nucleotides, and more preferably at least about 8 - 30 nucleotides corresponding to a region of the designated target nucleotide sequence. "Corresponding" means identical to or 20 complementary to the designated sequence. The oligonucleotide is not necessarily physically derived from any existing or natural sequence but may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription or a combination thereof.

The terms "oligonucleotide" or "nucleic acid" intend a polynucleotide of genomic DNA or RNA, cDNA, semi synthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is 25 not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature; and (3) is not found in nature. Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5'-phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbour in one direction via a phosphodiester linkage, an end of an 30 oligonucleotide is referred to as the "5<sup>1</sup> end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3<sup>1</sup> end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have a 5' and 3' ends. When two different, non-overlapping oligonucleotides anneal to different regions of the 35 same linear complementary nucleic acid sequence, the 3' end of one oligonucleotide points

toward the 5' end of the other; the former may be called the "upstream" oligonucleotide and the latter the "downstream" oligonucleotide.

By the term "SBC nucleobases" is meant "Selective Binding Complementary" nucleobases, i.e. modified nucleobases that can make stable hydrogen bonds to their complementary 5 nucleobases, but are unable to make stable hydrogen bonds to other SBC nucleobases. As an example, the SBC nucleobase A', can make a stable hydrogen bonded pair with its complementary unmodified nucleobase, T. Likewise, the SBC nucleobase T' can make a stable hydrogen bonded pair with its complementary unmodified nucleobase, A. However, the SBC nucleobases A' and T' will form an unstable hydrogen bonded pair as compared to the 10 base pairs A'-T and A-T'. Likewise, a SBC nucleobase of C is designated C and can make a stable hydrogen bonded pair with its complementary unmodified nucleobase G, and a SBC nucleobase of G is designated G' and can make a stable hydrogen bonded pair with its complementary unmodified nucleobase C, yet C and G' will form an unstable hydrogen bonded pair as compared to the base pairs C-G and C-G'. A stable hydrogen bonded pair is 15 obtained when 2 or more hydrogen bonds are formed e.g. the pair between A' and T, A and T', C and G', and C and G. An unstable hydrogen bonded pair is obtained when 1 or no hydrogen bonds is formed e.g. the pair between A' and T', and C and G'. Especially interesting SBC nucleobases are 2,6-diaminopurine (A', also called D) together with 2-thio-uracil (U', also called <sup>2S</sup>U)(2-thio-4-oxo-pyrimidine) and 2-thio-thymine (T', also called <sup>2S</sup>T)(2-thio-4-oxo-5-methyl-pyrimidine). Figure 4 in PCT Publication No. WO 2004/024314 illustrates 20 that the pairs A-<sup>2S</sup>T and D-T have 2 or more than 2 hydrogen bonds whereas the D-<sup>2S</sup>T pair forms a single (unstable) hydrogen bond. Likewise the SBC nucleobases pyrrolo-[2,3-d]pyrimidine-2(3H)-one (C, also called PyrroloPyr) and hypoxanthine (G', also called I)(6-oxo-purine) are shown in Figure 4 in PCT Publication No. WO 2004/024314 where the pairs 25 PyrroloPyr-G and C-I have 2 hydrogen bonds each whereas the PyrroloPyr-I pair forms a single hydrogen bond.

"SBC LNA oligomer" refers to a "LNA oligomer" containing at least one LNA monomer where the nucleobase is a "SBC nucleobase". By "LNA monomer with an SBC nucleobase" is meant a "SBC LNA monomer". Generally speaking SBC LNA oligomers include oligomers that besides 30 the SBC LNA monomer(s) contain other modified or naturally occurring nucleotides or nucleosides. By "SBC monomer" is meant a non-LNA monomer with a SBC nucleobase. By "isosequential oligonucleotide" is meant an oligonucleotide with the same sequence in a Watson-Crick sense as the corresponding modified oligonucleotide e.g. the sequences agTtcATg is equal to agTscD<sup>2S</sup>Ug where s is equal to the SBC DNA monomer 2-thio-t or 2-thio-u, D is equal to the SBC LNA monomer LNA-D and <sup>2S</sup>U is equal to the SBC LNA monomer LNA <sup>2S</sup>U. 35

The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5<sup>1</sup> end of one sequence is paired with the 3<sup>1</sup> end of the other, is in "antiparallel association." Bases not commonly found in natural nucleic acids may be included in the nucleic acids of the present invention include, for 5 example, inosine and 7-deazaguanine. Complementarity may not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, percent concentration of cytosine and guanine bases in the oligonucleotide, ionic strength, and incidence of mismatched base 10 pairs.

Stability of a nucleic acid duplex is measured by the melting temperature, or "T<sub>m</sub>". The T<sub>m</sub> of a particular nucleic acid duplex under specified conditions is the temperature at which half of the duplexes have disassociated.

The term "nucleobase" covers the naturally occurring nucleobases adenine (A), guanine (G), 15 cytosine (C), thymine (T) and uracil (U) as well as non-naturally occurring nucleobases such as xanthine, diaminopurine, 8-oxo-N<sup>6</sup>-methyladenine, 7-deazaxanthine, 7-deazaguanine, N<sup>4</sup>,N<sup>4</sup>-ethanocytosin, N<sup>6</sup>,N<sup>6</sup>-ethano-2,6-diaminopurine, 5-methylcytosine, 5~(C<sup>3</sup>-C<sup>6</sup>)-alkynyl-cytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridin, isocytosine, isoguanine, inosine and the "non-naturally occurring" 20 nucleobases described in Benner et al., U.S. Patent No. 5,432,272 and Susan M. Freier and Karl-Heinz Altmann, *Nucleic Acid Research*, 25: 4429-4443, 1997. The term "nucleobase" thus includes not only the known purine and pyrimidine heterocycles, but also heterocyclic analogues and tautomers thereof. Further naturally and non naturally occurring nucleobases include those disclosed in U.S. Patent No. 3,687,808; in chapter 15 by Sanghvi, in *Antisense 25 Research and Application*, Ed. S. T. Crooke and B. Lebleu, CRC Press, 1993; in Englisch, et al., *Angewandte Chemie, International Edition*, 30: 613-722, 1991 (see, especially pages 622 and 623, and in the *Concise Encyclopedia of Polymer Science and Engineering*, J. I. Kroschwitz Ed., John Wiley & Sons, pages 858-859, 1990, Cook, *Anti-Cancer DrugDesign* 6: 585-607, 1991, each of which are hereby incorporated by reference in their entirety).

30 The term "nucleosidic base" or "nucleobase analogue" is further intended to include heterocyclic compounds that can serve as like nucleosidic bases including certain "universal bases" that are not nucleosidic bases in the most classical sense but serve as nucleosidic bases. Especially mentioned as a universal base is 3-nitropyrrole or a 5-nitroindole. Other preferred compounds include pyrene and pyridyloxazole derivatives, pyrenyl, 35 pyrenylmethylglycerol derivatives and the like. Other preferred universal bases include, pyrrole, diazole or triazole derivatives, including those universal bases known in the art.

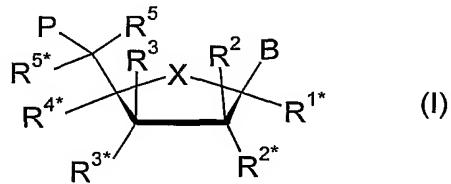
By "oligonucleotide," "oligomer," or "oligo" is meant a successive chain of monomers (e.g., glycosides of heterocyclic bases) connected via internucleoside linkages. The linkage between two successive monomers in the oligo consist of 2 to 4, desirably 3, groups/atoms selected from  $-\text{CH}_2-$ ,  $-\text{O}-$ ,  $-\text{S}-$ ,  $-\text{NR}^{\text{H}}$ ,  $>\text{C}=\text{O}$ ,  $>\text{C}=\text{NR}^{\text{H}}$ ,  $>\text{C}=\text{S}$ ,  $-\text{Si}(\text{R}^{\text{H}})_2-$ ,  $-\text{SO}-$ ,  $-\text{S}(\text{O})_2-$ ,  
5  $-\text{P}(\text{O})_2-$ ,  $-\text{PO}(\text{BH}_3)-$ ,  $-\text{P}(\text{O},\text{S})-$ ,  $-\text{P}(\text{S})_2-$ ,  $-\text{PO}(\text{R}^{\text{H}})-$ ,  $-\text{PO}(\text{OCH}_3)-$ , and  $-\text{PO}(\text{NHR}^{\text{H}})-$ , where  $\text{R}^{\text{H}}$  is selected from hydrogen and  $\text{C}_{1-4}\text{-alkyl}$ , and  $\text{R}^{\text{H}}$  is selected from  $\text{C}_{1-6}\text{-alkyl}$  and phenyl. Illustrative examples of such linkages are  $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ ,  $-\text{CH}_2-\text{CO-CH}_2-$ ,  $-\text{CH}_2-\text{CHOH-CH}_2-$ ,  $-\text{O-CH}_2-\text{O-}$ ,  $-\text{O-CH}_2-\text{CH}_2-$ ,  $-\text{O-CH}_2-\text{CH=}$  (including  $\text{R}^{\text{H}}$  when used as a linkage to a succeeding monomer),  $-\text{CH}_2-\text{CH}_2-\text{O-}$ ,  $-\text{NR}^{\text{H}}-\text{CH}_2-\text{CH}_2-$ ,  $-\text{CH}_2-\text{CH}_2-\text{NR}^{\text{H}}-$ ,  $-\text{CH}_2-\text{NR}^{\text{H}}-\text{CH}_2-$ ,  $-\text{O-CH}_2-\text{CH}_2-\text{NR}^{\text{H}}-$ ,  
10  $-\text{NR}^{\text{H}}-\text{CO-O-}$ ,  $-\text{NR}^{\text{H}}-\text{CO-NR}^{\text{H}}-$ ,  $-\text{NR}^{\text{H}}-\text{CS-NR}^{\text{H}}-$ ,  $-\text{NR}^{\text{H}}-\text{C}(\text{=NR}^{\text{H}})-\text{NR}^{\text{H}}-$ ,  $-\text{NR}^{\text{H}}-\text{CO-CH}_2-\text{NIR}^{\text{H}}-$ ,  $-\text{O-CO-}$ ,  $-\text{O-CO-CH}_2-\text{O-}$ ,  $-\text{O-CH}_2-\text{CO-O-}$ ,  $-\text{CH}_2-\text{CO-NR}^{\text{H}}-$ ,  $-\text{O-CO-NR}^{\text{H}}-$ ,  $-\text{NR}^{\text{H}}-\text{CO-CH}_2-$ ,  $-\text{O-CH}_2-\text{CO-}$ ,  $-\text{NR}^{\text{H}}-$ ,  $-\text{O-CH}_2-\text{CH}_2-\text{NR}^{\text{H}}-$ ,  $-\text{CH}=\text{N-O-}$ ,  $-\text{CH}_2-\text{NR}^{\text{H}}-\text{O-}$ ,  $-\text{CH}_2-\text{O-N=}$  (including  $\text{R}^{\text{H}}$  when used as a linkage to a succeeding monomer),  $-\text{CH}_2-\text{O-NRS-}$   $-\text{CO-NR}^{\text{H}}-\text{CH}_2-$ ,  $-\text{CH}_2-\text{NR}^{\text{H}}-\text{O-}$ ,  $-\text{CH}_2-\text{NR}^{\text{H}}-\text{CO-}$ ,  $-\text{O-NR}^{\text{H}}-\text{CH}_2-$ ,  $-\text{O-NR}^{\text{H}}-$ ,  $-\text{O-CH}_2-\text{S-}$ ,  $-\text{S-CH}_2-\text{O-}$ ,  $-\text{CH}_2-\text{CH}_2-\text{S-}$ ,  $-\text{O-CH}_2-\text{CH}_2-\text{S-}$ ,  $-\text{S-CH}_2-\text{CH=}$   
15 (including  $\text{R}^{\text{H}}$  when used as a linkage to a succeeding monomer),  $-\text{S-CH}_2-\text{CH}_2-$ ,  $-\text{S-CH}_2-\text{CH}_2-\text{O-}$ ,  $-\text{S-CH}_2-\text{CH}_2-\text{S-}$ ,  $-\text{CH}_2-\text{S-CH}_2-$ ,  $-\text{CH}_2-\text{SO-CH}_2-$ ,  $-\text{CH}_2-\text{SO}_2-\text{CH}_2-$ ,  $-\text{O-SO-0-}$ ,  $-\text{O-S}(\text{O})_2-\text{O-}$ ,  $-\text{O-S}(\text{O})_2-\text{NR}^{\text{H}}-$ ,  $-\text{NR}^{\text{H}}-\text{S}(\text{O})_2-\text{CH}_2-$ ,  $-\text{O-S}(\text{O})_2-\text{CH}_2-$ ,  $-\text{O-P}(\text{O})_2-\text{O-}$ ,  $-\text{O-P}(\text{O},\text{S})-\text{O-}$ ,  $-\text{O-P}(\text{S})_2-\text{O-}$ ,  $-\text{S-P}(\text{O})_2-\text{O-}$ ,  $-\text{S-P}(\text{CS})-\text{O-}$ ,  $-\text{S-P}(\text{S})_2-\text{O-}$ ,  $-\text{O-P}(\text{O})_2-\text{S-}$ ,  $-\text{O-P}(\text{O},\text{S})-\text{S-}$ ,  $-\text{O-P}(\text{S})_2-\text{S-}$ ,  $-\text{S-P}(\text{O})_2-\text{S-}$ ,  $-\text{S-P}(\text{O},\text{S})-\text{S-}$ ,  $-\text{S-P}(\text{S})_2-\text{S-}$ ,  $-\text{O-PO}(\text{R}^{\text{H}}-\text{J-O-})$ ,  $-\text{O-PO}(\text{OCH}_3)-\text{O-}$ ,  $-\text{O-PO}(\text{OCH}_2\text{CH}_3)-\text{O-}$ ,  
20  $-\text{O-PO}(\text{OCH}_2\text{CH}_2\text{S-R})-\text{O-}$ ,  $-\text{O-PO}(\text{BH}_3\text{J-O-})$ ,  $-\text{O-PO}(\text{NHR}^{\text{H}})-\text{O-}$ ,  $-\text{O-P}(\text{O})_2-\text{NR}^{\text{H}}-$ ,  $-\text{NR}^{\text{H}}-\text{P}(\text{O})_2-\text{O-}$ ,  $-\text{O-P}(\text{O},\text{NR}^{\text{H}})-\text{O-}$ ,  $-\text{CH}_2-\text{P}(\text{O})_2-\text{O-}$ ,  $-\text{O-P}(\text{O})_2-\text{CH}_2-$ , and  $-\text{O-Si}(\text{R}^{\text{H}}-\text{J}_2-\text{O-})$ ; among which  $-\text{CH}_2-\text{CO-}$ ,  $-\text{CH}_2-\text{NR}^{\text{H}}-\text{O-}$ ,  $-\text{S-CH}_2-\text{O-}$ ,  $-\text{O-P}(\text{O})_2-\text{O-}$ ,  $-\text{O-P}(\text{O},\text{S})-\text{O-}$ ,  $-\text{O-P}(\text{S})_2-\text{O-}$ ,  $-\text{NR}^{\text{H}}-\text{P}(\text{O})_2-\text{O-}$ ,  $-\text{O-P}(\text{O},\text{NR}^{\text{H}})-\text{O-}$ ,  $-\text{O-PO}(\text{R}^{\text{H}}-\text{J-O-})$ ,  $-\text{O-PO}(\text{CH}_3\text{J-O-})$ , and  $-\text{O-PO}(\text{NHR}^{\text{H}})-\text{O-}$ , where  $\text{R}^{\text{H}}$  is selected from hydrogen and  $\text{C}_{1-4}\text{-alkyl}$ , and  $\text{R}^{\text{H}}$  is selected from  $\text{C}_{1-6}\text{-alkyl}$  and phenyl, are especially  
25 desirable. Further illustrative examples are given in Mesmaeker *et al.*, Current Opinion in Structural Biology 1995, 5, 343-355 and Susan M. Freier and Karl-Heinz Altmann, Nucleic Acids Research, 1997, vol 25, pp 4429-4443. The left-hand side of the internucleoside linkage is bound to the 5-membered ring as substituent  $\text{P}^{\text{r}}$  at the 3'-position, whereas the right-hand side is bound to the 5'-position of a preceding monomer.

30 By "LNA" or "LNA monomer" (e.g., an LNA nucleoside or LNA nucleotide) or an LNA oligomer (e.g., an oligonucleotide or nucleic acid) is meant a nucleoside or nucleotide analogue that includes at least one LNA monomer. LNA monomers as disclosed in PCT Publication WO 99/14226 are in general particularly desirable modified nucleic acids for incorporation into an oligonucleotide of the invention. Additionally, the nucleic acids may be modified at either  
35 the 3' and/or 5' end by any type of modification known in the art. For example, either or both ends may be capped with a protecting group, attached to a flexible linking group, attached to a reactive group to aid in attachment to the substrate surface, etc. Desirable LNA monomers and their method of synthesis also are disclosed in US 6,043,060, US

6,268,490, PCT Publications WO 01/07455, WO 01/00641, WO 98/39352, WO 00/56746, WO 00/56748 and WO 00/66604 as well as in the following papers: Morita *et al.*, Bioorg. Med. Chem. Lett. 12(1):73-76, 2002; Hakansson *et al.*, Bioorg. Med. Chem. Lett. 11(7):935-938, 2001; Koshkin *et al.*, J. Org. Chem. 66(25):8504-8512, 2001; Kvaemo *et al.*, J. Org. Chem. 66(16):5498-5503, 2001; Hakansson *et al.*, J. Org. Chem. 65(17):5161-5166, 2000; Kvaerno *et al.*, J. Org. Chem. 65(17):5167-5176, 2000; Pfundheller *et al.*, Nucleosides Nucleotides 18(9):2017-2030, 1999; and Kumar *et al.*, Bioorg. Med. Chem. Lett. 8(16):2219-2222, 1998.

Preferred LNA monomers, also referred to as "oxy-LNA" are LNA monomers which include 10 bicyclic compounds as disclosed in PCT Publication WO 03/020739 wherein the bridge between R<sup>4</sup> and R<sup>2</sup> as shown in formula (I) below together designate -CH<sub>2</sub>-O- or -CH<sub>2</sub>-CH<sub>2</sub>-O-.

By "LNA modified oligonucleotide" or "LNA substituted oligonucleotide" is meant a 15 oligonucleotide comprising at least one LNA monomer of formula (I), described *infra*, having the below described illustrative examples of modifications:



wherein X is selected from -O-, -S-, -N(R<sup>N</sup>)-, -C(R<sup>6</sup>R<sup>6\*</sup>)-, -O-C(R<sup>7</sup>R<sup>7\*</sup>)-, -C(R<sup>6</sup>R<sup>6\*</sup>)-O-, -S-C(R<sup>7</sup>R<sup>7\*</sup>)-, -C(R<sup>6</sup>R<sup>6\*</sup>)-S-, -N(R<sup>N</sup>)-C(R<sup>7</sup>R<sup>7\*</sup>)-, -C(R<sup>6</sup>R<sup>6\*</sup>)-N(R<sup>N</sup>)-, and -C(R<sup>6</sup>R<sup>6\*</sup>)-C(R<sup>7</sup>R<sup>7\*</sup>).

B is selected from a modified base as discussed above e.g. an optionally substituted 20 carbocyclic aryl such as optionally substituted pyrene or optionally substituted pyrenylmethylglycerol, or an optionally substituted heteroalicyclic or optionally substituted heteroaromatic such as optionally substituted pyridyloxazole, optionally substituted pyrrole, optionally substituted diazole or optionally substituted triazole moieties; hydrogen, hydroxy, optionally substituted C<sub>1-4</sub>-alkoxy, optionally substituted C<sub>1-4</sub>-alkyl, optionally substituted C<sub>1-4</sub>-acyloxy, nucleobases, DNA intercalators, photochemically active groups, thermochemically 25 active groups, chelating groups, reporter groups, and ligands.

P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group, such internucleoside linkage or 5'-terminal group optionally including the substituent R<sup>5</sup>. One of the substituents R<sup>2</sup>, R<sup>2\*</sup>, R<sup>3</sup>, and R<sup>3\*</sup> is a group P\* which designates an

internucleoside linkage to a preceding monomer, or a 2'/3'-terminal group. The substituents of R<sup>1\*</sup>, R<sup>4\*</sup>, R<sup>5</sup>, R<sup>5\*</sup>, R<sup>6</sup>, R<sup>6\*</sup>, R<sup>7</sup>, R<sup>7\*</sup>, R<sup>N</sup>, and the ones of R<sup>2</sup>, R<sup>2\*</sup>, R<sup>3</sup>, and R<sup>3\*</sup> not designating P\* each designates a biradical comprising about 1-8 groups/atoms selected from -C(R<sup>a</sup>R<sup>b</sup>)-, -C(R<sup>a</sup>)=C(R<sup>a</sup>)-, -C(R<sup>a</sup>)=N-, -C(R<sup>a</sup>)-O-, -O-, -Si(R<sup>a</sup>)<sub>2</sub>~, -C(R<sup>a</sup>)-S-, -S-, -SO<sub>2</sub>-, -C(R<sup>a</sup>)-N(R<sup>b</sup>)-, -N(R<sup>a</sup>)-, and >C=Q, wherein Q is selected from -O-, -S-, and -N(R<sup>a</sup>)-, and R<sup>a</sup> and R<sup>b</sup> each is independently selected from hydrogen, optionally substituted Ci-i<sub>2</sub>-all<yl, optionally substituted C<sub>2</sub>-i<sub>2</sub>-alkenyl, optionally substituted C<sub>2</sub>-i<sub>2</sub>-alkynyl, hydroxy, Q-alkoxy, C<sub>2-12</sub>-alkenyloxy, carboxy, C<sub>1-12</sub>-alkoxycarbonyl, Ci-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, hetero-aryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C<sub>1-6</sub>-alkyl)amino, carbamoyl, mono- and di(C<sub>1-6</sub>-alkyl)-amino-carbonyl, amino-Ci-alkyl-aminocarbonyl, mono- and di(Ci-alkyl)amino-Ci-alkyl-aminocarbonyl, Ci-g-alkyl-carbonylamino, carbamido, Ci-alkanoyloxy, sulphono, C<sub>1-6</sub>-alkylsulphonyloxy, nitro, azido, sulphanyl, C<sub>1-6</sub>-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted, and where two geminal substituents R<sup>a</sup> and R<sup>b</sup> together may designate optionally substituted methylene (=CH<sub>2</sub>), and wherein two non-geminal or geminal substituents selected from R<sup>a</sup>, R<sup>b</sup>, and any of the substituents R<sup>1\*</sup>, R<sup>2</sup>, R<sup>2\*</sup>, R<sup>3</sup>, R<sup>3\*</sup>, R<sup>4\*</sup>, R<sup>5</sup>, R<sup>5\*</sup>, R<sup>6</sup> and R<sup>6\*</sup>, R<sup>7</sup>, and R<sup>7\*</sup> which are present and not involved in P, P\* or the biradical(s) together may form an associated biradical selected from biradicals of the same kind as defined before; the pair(s) of non-geminal substituents thereby forming a mono- or bicyclic entity together with (i) the atoms to which said non-geminal substituents are bound and (ii) any intervening atoms.

Each of the substituents R<sup>1\*</sup>, R<sup>2</sup>, R<sup>2\*</sup>, R<sup>3</sup>, R<sup>4\*</sup>, R<sup>5</sup>, R<sup>5\*</sup>, R<sup>6</sup> and R<sup>6\*</sup>, R<sup>7</sup>, and R<sup>7\*</sup> which are present and not involved in P, P\* or the biradical(s), is independently selected from hydrogen, optionally substituted Ci-alkyl, optionally substituted C<sub>2-12</sub>-alkenyl, optionally substituted C<sub>2</sub>-i<sub>2</sub>-alkynyl, hydroxy, Ci-alkoxy, C<sub>2-12</sub>-alkenyloxy, carboxy, Ci-i<sub>2</sub>-alkoxycarbonyl, C<sub>1-12</sub>-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C<sub>1-6</sub>-alkyl)amino, carbamoyl, mono- and di(C<sub>1-6</sub>-alkyl)-amino-carbonyl, amino-Ci-alkyl-aminocarbonyl, mono- and diCCi-e-alkyOamino-Ci-e-alkyl-aminocarbonyl, C<sub>1-5</sub>-alkyl-carbonylamino, carbamido, Ci-alkanoyloxy, sulphono, C<sub>1-6</sub>-alkylsulphonyloxy, nitro, azido, sulphanyl, C<sub>1-6</sub>-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted, and where two geminal substituents together may designate oxo, thioxo, imino, or optionally substituted methylene, or together may form a spiro biradical consisting of a 1-5 carbon atom(s) alkylene chain which is optionally interrupted and/or terminated by one or more heteroatoms/groups selected from -O-, -S-, and -(NR<sup>N</sup>)- where R<sup>N</sup> is selected from hydrogen and C<sub>1-4</sub>-alkyl, and where two adjacent (non-

geminal) substituents may designate an additional bond resulting in a double bond; and  $R^N$ , when present and not involved in a biradical, is selected from hydrogen and  $d_{-4}$ -alkyl; and basic salts and acid addition salts thereof.

Exemplary 5', 3", and/or 2' terminal groups include -H, -OH, halo (e.g., chloro, fluoro, iodo, or bromo), optionally substituted aryl, (e.g., phenyl or benzyl), alkyl (e.g., methyl or ethyl), alkoxy (e.g., methoxy), acyl (e.g. acetyl or benzoyl), aroyl, aralkyl, hydroxy, hydroxyalkyl, alkoxy, aryloxy, aralkoxy, nitro, cyano, carboxy, alkoxycarbonyl, aryloxycarbonyl, aralkoxycarbonyl, acylamino, aroylamino, alkylsulfonyl, arylsulfonyl, heteroarylssulfonyl, alkylsulfinyl, arylsulfinyl, heteroarylssulfinyl, alkylthio, arylthio, heteroarylthio, aralkylthio, heteroaralkylthio, amidino, amino, carbamoyl, sulfamoyl, alkene, alkyne, protecting groups (e.g., silyl, 4,4'-dimethoxytrityl, monomethoxytrityl, or trityl(triphenylmethyl)), linkers (e.g., a linker containing an amine, ethylene glycol, quinone such as anthraquinone), detectable labels (e.g., radiolabels or fluorescent labels), and biotin.

It is understood that references herein to a nucleic acid unit, nucleic acid residue, LNA monomer, or similar term are inclusive of both individual nucleoside units and nucleotide units and nucleoside units and nucleotide units within an oligonucleotide.

A "modified base" or other similar terms refer to a composition (e.g., a non-naturally occurring nucleobase or nucleosidic base), which can pair with a natural base (e.g., adenine, guanine, cytosine, uracil, and/or thymine) and/or can pair with a non-naturally occurring nucleobase or nucleosidic base. Desirably, the modified base provides a  $T_m$  differential of 15, 12, 10, 8, 6, 4, or 2 $^{\circ}$ C or less as described herein. Exemplary modified bases are described in EP 1 072 679 and WO 97/12896.

The term "chemical moiety" refers to a part of a molecule. "Modified by a chemical moiety" thus refer to a modification of the standard molecular structure by inclusion of an unusual chemical structure. The attachment of said structure can be covalent or non-covalent.

The term "inclusion of a chemical moiety" in an oligonucleotide probe thus refers to attachment of a molecular structure. Such as chemical moiety include but are not limited to covalently and/or non-covalently bound minor groove binders (MGB) and/or intercalating nucleic acids (INA) selected from a group consisting of asymmetric cyanine dyes, DAPI, SYBR Green I, SYBR Green II, SYBR Gold, PicoGreen, thiazole orange, Hoechst 33342, Ethidium Bromide, 1-O-(1-pyrenylmethyl)glycerol and Hoechst 33258. Other chemical moieties include the modified nucleobases, nucleosidic bases or LNA modified oligonucleotides.

"Oligonucleotide analogue" refers to a nucleic acid binding molecule capable of recognizing a particular target nucleotide sequence. A particular oligonucleotide analogue is peptide nucleic acid (PNA) in which the sugar phosphate backbone of an oligonucleotide is replaced by a protein like backbone. In PNA, nucleobases are attached to the uncharged polyamide backbone yielding a chimeric pseudopeptide-nucleic acid structure, which is homomorphous to nucleic acid forms.

"High affinity nucleotide analogue" or "affinity-enhancing nucleotide analogue" refers to a non-naturally occurring nucleotide analogue that increases the "binding affinity" of an oligonucleotide probe to its complementary recognition sequence when substituted with at least one such high-affinity nucleotide analogue.

As used herein, a probe with an increased "binding affinity" for a recognition sequence compared to a probe which comprises the same sequence but does not comprise a stabilizing nucleotide, refers to a probe for which the association constant ( $K_a$ ) of the probe recognition segment is higher than the association constant of the complementary strands of a double-stranded molecule. In another preferred embodiment, the association constant of the probe recognition segment is higher than the dissociation constant ( $K_d$ ) of the complementary strand of the recognition sequence in the target sequence in a double stranded molecule.

Monomers are referred to as being "complementary" if they contain nucleobases that can form hydrogen bonds according to Watson-Crick base-pairing rules (e.g. G with C, A with T or A with U) or other hydrogen bonding motifs such as for example diaminopurine with T, 5-methyl C with G, 2-thiothymidine with A, inosine with C, pseudouridine with G, etc.

The term "succeeding monomer" relates to the neighbouring monomer in the 5'-terminal direction and the "preceding monomer" relates to the neighbouring monomer in the 3'-terminal direction.

The term "target nucleic acid" or "target ribonucleic acid" refers to any relevant nucleic acid of a single specific sequence, e. g., a biological nucleic acid, e. g., derived from a patient, an animal (a human or non-human animal), a plant, a bacteria, a fungi, an archaea, a cell, a tissue, an organism, etc. For example, where the target ribonucleic acid or nucleic acid is derived from a bacteria, archaea, plant, non-human animal, cell, fungi, or non-human organism, the method optionally further comprises selecting the bacteria, archaea, plant, non-human animal, cell, fungi, or non-human organism based upon detection of the target nucleic acid. In one embodiment, the target nucleic acid is derived from a patient, e.g., a human patient. In this embodiment, the invention optionally further includes selecting a treatment,

diagnosing a disease, or diagnosing a genetic predisposition to a disease, based upon detection of the target nucleic acid.

"Target sequence" refers to a specific nucleic acid sequence within any target nucleic acid.

The term "stringent conditions", as used herein, is the "stringency" which occurs within a range from about  $T_m$ -5° C. (5° C. below the melting temperature ( $T_m$ ) of the probe) to about 20° C. to 25° C. below  $T_m$ . As will be understood by those skilled in the art, the stringency of hybridization may be altered in order to identify or detect identical or related polynucleotide sequences. Hybridization techniques are generally described in *Nucleic Acid Hybridization, A Practical Approach*, Ed. Hames, B. D. and Higgins, S. J., IRL Press, 1985; Gall and Pardue, *Proc. Natl. Acad. Sci., USA* 63: 378-383, 1969; and John, et al. *Nature* 223: 582-587, 1969.

#### Detailed Description of the Invention

##### *Collection of probes of the invention*

As briefly stated above, the probe collections or libraries of the present invention are so designed each member of said collection comprises a recognition sequence consisting of nucleobases and affinity enhancing nucleobase analogues, and wherein the recognition sequences exhibit a combination of high melting temperatures and low self-complementarity scores, said melting temperatures being the melting temperature of the duplex between the recognition sequence and its complementary DNA or RNA sequence.

This design provides for probes which are highly specific for their target sequences but which at the same time exhibits a very low risk of self-annealing (as evidenced by a low self-complementarity score) - self-annealing is, due to the presence of affinity enhancing nucleobases (such as LNA monomers) a problem which is more serious than when using conventional deoxyribonucleotide probes.

In one embodiment the recognition sequences exhibit a melting temperature (or a measure of melting temperature) corresponding to at least 5°C higher than a melting temperature or a measure of melting temperature of the self-complementarity score under conditions where the probe hybridizes specifically to its complementary target sequence (alternatively, one can quantify the "risk of self-annealing" feature by requiring that the melting temperature of the probe-target duplex must be at least 5°C higher than the melting temperature of duplexes between the probes or the probes internally). The collection may be so constituted that at least 90% (such as at least 95%) of the recognition sequences exhibit a melting temperature or a measure of melting temperature corresponding to at least 5°C higher than a melting

temperature or a measure of melting temperature of the self-complementarity score under conditions where the probe hybridizes specifically to its complementary target sequence (or that at least the same percentages of probes exhibit a melting temperature of the probe-target duplex of at least 5°C more than the melting temperature of duplexes between the probes or the probes internally). In a preferred embodiment all of the detection probes include recognition sequences which exhibit a melting temperature or a measure of melting temperature corresponding to at least 5°C higher than a melting temperature or a measure of melting temperature of the self-complementarity score under conditions where the probe hybridizes specifically to its complementary target sequence.

10 However, it is preferred that this temperature difference is higher, such as at least 10°C, such as at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, and at least 50°C higher than a melting temperature or measure of melting temperature of the self-complementarity score.

15 In one embodiment a collection of probes according to the present invention comprises at least 10 detection probes, 15 detection probes, such as at least 20, at least 25, at least 50, at least 75, at least 100, at least 200, at least 500, at least 1000, and at least 2000 members.

It is preferred that the collection of probes of the invention is capable of specifically detecting all or substantially all members of the transcriptome of an organism.

20 In another preferred embodiment, the collection of probes is capable of specifically detecting all small non-coding RNAs of an organism, such as all miRNAs or siRNAs.

25 The organism is selected from the group consisting of a bacterium, a yeast, a fungus, a protozoan, a plant, and an animal. Specific examples of genera and species of such organisms are mentioned herein, and the inventive collection of probes may be designed for all of these specific genera and species.

30 In one embodiment, the affinity-enhancing nucleobase analogues are regularly spaced between the nucleobases in at least 80% of the members of said collection, such as in at least 90% or at least 95% of said collection (in one embodiment, all members of the collection contain regularly spaced affinity-enhancing nucleobase analogues). One reason for this is that the time needed for adding each nucleobase or analogue during synthesis of the probes of the invention is dependent on whether or not a nucleobase analogue is added. By using the "regular spacing strategy" considerable production benefits are achieved. Specifically for LNA nucleobases, the required coupling times for incorporating LNA amidites

5 during synthesis may exceed that required for incorporating DNA amidites. Hence, in cases involving simultaneous parallel synthesis of multiple oligonucleotides on the same instrument, it is advantageous if the nucleotide analogues such as LNA are spaced evenly in the same pattern as derived from the 3'-end, to allow reduced cumulative coupling times for the synthesis. The affinity enhancing nucleobase analogues are conveniently regularly spaced as every 2<sup>nd</sup>, every 3<sup>rd</sup>, every 4<sup>th</sup> or every 5<sup>th</sup> nucleobase in the recognition sequence, and preferably as every 3<sup>rd</sup> nucleobase.

In one embodiment of the the collection of probes, all members contain affinity enhancing nucleobase analogues with the same regular spacing in the recognition sequences.

- 10 The presence of the affinity enhancing nucleobases in the recognition sequence preferably confers an increase in the binding affinity between a probe and its complementary target nucleotide sequence relative to the binding affinity exhibited by a corresponding probe, which only include nucleobases. Since LNA nucleobases/monomers have this ability, it is preferred that the affinity enhancing nucleobase analogues are LNA nucleobases.
- 15 In some embodiments, the 3' and 5' nucleobases are not substituted by affinity enhancing nucleobase analogues.

As detailed herein, one huge advantage of the probes of the invention is their short lengths which surprisingly provides for high target specificity and advantages in detecting small RNAs and detecting nucleic acids in samples not normally suitable for hybridization detection

- 20 strategies. It is, however, preferred that the probes comprise a recognition sequence is at least a 6-mer, such as at least a 7-mer, at least an 8-mer, at least a 9-mer, at least a 10-mer, at least an 11-mer, at least a 12-mer, at least a 13-mer, at least a 14-mer, at least a 15-mer, at least a 16-mer, at least a 17-mer, at least an 18-mer, at least a 19-mer, at least a 20-mer, at least a 21-mer, at least a 22-mer, at least a 23-mer, and at least a 24-mer. On the other hand, the recognition sequence is preferably at most a 25-mer, such as at most a 24-mer, at most a 23-mer, at most a 22-mer, at most a 21-mer, at most a 20-mer, at most a 19-mer, at most an 18-mer, at most a 17-mer, at most a 16-mer, at most a 15-mer, at most a 14-mer, at most a 13-mer, at most a 12-mer, at most an 11-mer, at most a 10-mer, at most a 9-mer, at most an 8-mer, at most a 7-mer, and at most a 6-mer.
- 30 Also for production purposes, it is an advantage that a majority of the probes in a collection are of the same length. In preferred embodiments, the collection of probes of the invention is one wherein at least 80% of the members comprise recognition sequences of the same length, such as at least 90% or at least 95%.

As discussed above, it is advantageous, in order to avoid self-annealing, that at least one of the nucleobases in the recognition sequence is substituted with its corresponding selectively binding complementary (SBC) nucleobase.

Typically, the nucleobases in the sequence are selected from ribonucleotides and  
5 deoxyribonucleotides, preferably deoxyribonucleotides. It is preferred that the recognition sequence consists of affinity enhancing nucleobase analogues together with either ribonucleotides or deoxyribonucleotides.

In certain embodiments, each member of a collection is covalently bonded to a solid support.  
Such a solid support may be selected from a bead, a microarray, a chip, a strip, a  
10 chromatographic matrix, a microtiter plate, a fiber or any other convenient solid support generally accepted in the art in order to facilitate the exercise of the methods discussed generally and specifically

As also detailed herein, each detection probe in a collection of the invention may include a detection moiety and/or a ligand, optionally placed in the recognition sequence but also  
15 placed outside the recognition sequence. The detection probe may thus include a photochemically active group, a thermochemically active group, a chelating group, a reporter group, or a ligand that facilitates the direct or indirect detection of the probe or the immobilisation of the oligonucleotide probe onto a solid support.

*Probes of the invention*

20 The present invention provides novel oligonucleotide compositions and probe sequences for the use in detection, isolation, purification, amplification, identification, quantification, or capture of miRNAs, their target mRNAs, stem-loop precursor miRNAs, siRNAs, other non-coding RNAs, RNA-edited transcripts or alternative mRNA splice variants or single stranded DNA (e.g. viral DNA) characterized in that the probe sequences contain a number of  
25 nucleoside analogues.

In a preferred embodiment the number of nucleoside analogue corresponds to from 20 to 40% of the oligonucleotide of the invention.

In a preferred embodiment the probe sequences are substituted with a nucleoside analogue with regular spacing between the substitutions

30 In another preferred embodiment the probe sequences are substituted with a nucleoside analogue with irregular spacing between the substitutions

In a preferred embodiment the nucleoside analogue is LNA.

In a further preferred embodiment the detection probe sequences comprise a photochemically active group, a thermochemically active group, a chelating group, a reporter group, or a ligand that facilitates the direct or indirect detection of the probe or the

5 immobilisation of the oligonucleotide probe onto a solid support.

In a further preferred embodiment:

(a) the photochemically active group, the thermochemically active group, the chelating group, the reporter group, or the ligand includes a spacer (K), said spacer comprising a chemically cleavable group; or

10 (b) the photochemically active group, the thermochemically active group, the chelating group, the reporter group, or the ligand is attached via the biradical of at least one of the LNA(s) of the oligonucleotide.

Especially preferred detection probes of the invention are those that include the LNA containing recognition sequences set forth in tables A-K, 1, 3 and 15-1 herein.

15 *Methods for defining and preparing probes an probe collections*

The invention relates to a method for expanding or building a collection defined above, comprising

A) defining a reference nucleotide sequence consisting of nucleobases, said reference nucleotide sequence being complementary to a target sequence for which the collection does

20 not contain a detection probe,

B) substituting the reference nucleotide sequence's nucleobases with affinity enhancing nucleobase analogues to provide a set of chimeric sequences wherein,

C) determining usefulness of each of the chimeric sequences based on assessment of their ability to self-anneal and their melting temperature, and

25 D) synthesizing and adding, to the collection, a probe comprising as its recognition sequence the chimeric sequence with the optimum combination of high melting temperature and low self-annealing.

In order to ensure that the optimum probes are added to the library, step B preferably includes provision of all possible chimeric sequences which include a particular set of affinity

30 enhancing nucleobase analogues. By this is meant that prior to exercise of the method, it is decided which affinity enhancing nucleobases should be used in the design phase (typically

one for each of the 4 naturally occurring nucleobases). After this choice has been made, step B runs through an iterative process in order to define all possible chimeric sequences. In order to reduce the comprehensive nature of this step, it can also be decided to utilize the "regular spacing" strategy referred to above, since this will inherently reduce the number of 5 chimeric sequences to evaluate in step C. So, basically this means that only chimeric sequences, wherein the affinity enhancing nucleobase analogues are regularly spaced between the nucleobases, are added to the collection in step D.

Step C comprises the herein-discussed evaluation of melting temperature differences of at least 5°C between melting temperature for the duplex between the potential probe and its target 10 target and the melting temperature characterizing self-annealing. Hence, all disclosures relating to these preferred differences in melting temperature referred to above in the discussion of the probe collections apply mutatis mutandis to the determination in step C.

Preferably, the melting temperature difference used for the determination in step C is at least 15°C.

15 Apart from that, all disclosures relating to the characteristics of the probes in the collections of the invention apply mutatis mutandis to the above referenced method, meaning that the probes designed/produced may further include all the features characterizing the probes of the present invention.

A similar method may be utilized to design single probes, comprising  
20 1) defining a reference nucleotide sequence consisting of nucleobases, said reference nucleotide sequence being complementary to said target nucleotide sequence,  
2) substituting the reference nucleotide sequence's nucleobases with affinity enhancing nucleobase analogues to provide a set of chimeric sequences  
3) determining usefulness of each of the chimeric sequences based on assessment of their 25 ability to self-anneal and their melting temperatures, and  
4) defining the optimized detection probe as the one in the set having as its recognition sequence the chimeric sequence with the optimum combination of high melting temperature and low self-annealing.

As above, step 2 may include provision of all possible chimeric sequences which include a 30 particular set of affinity enhancing nucleobase analogues and as above only chimeric sequences, wherein the affinity enhancing nucleobase analogues are regularly spaced between the nucleobases, are defined in step 4 or, if applicable, are synthesized - this is because the method may also entail synthesizing the optimized detection probe. And, in general, all disclosures herein relating to the characteristics of the probes in the collections of

the invention apply mutatis mutandis to the above referenced method for design of single probes, meaning that the probes designed/produced may further include all the features characterizing the probes of the present invention. This e.g. includes that the detection probe may be further modified by containing at least one SBC nucleobase as one of the 5 nucleobases, and in general, the detection probe designed may be any detection probe disclosed herein.

Both of the above-referenced methods may be performed partly in silico, *i.e.* all steps relating to the design phase. Since sequence alignments and melting temperature calculations may be accomplished by the use of software, the present methods are preferably 10 exercised at least partially in a software environment. That is, above-referenced steps A-C or 1-4, may be performed in silico and the invention also relates to a computer system comprising a computer program product/executable code which can perform such a method.

Hence, the present invention also relates to a computer system for designing an optimized detection probe for a target nucleic acid sequence, said system comprising 15 a) input means for inputting the target nucleotide (can be a manual input interface such as a keyboard but conveniently simple queries in a database or input from a source file) b) storage means for storing the target nucleotide sequence (RAM, a harddisk or any other suitable volatile memory), c) optionally executable code which can calculate a reference nucleotide sequence being 20 complementary to said target nucleotide sequence and/or input means for inputting the reference nucleotide sequence, d) optionally storage means for storing the reference nucleotide sequence (features c and d are optional because these, although convenient, are not necessary in order to create a chimeric sequence, cf. next step), 25 e) executable code which can generate chimeric sequences from the reference nucleotide sequence or the target nucleic acid sequence, wherein said chimeric sequences comprise the reference nucleotide sequence, wherein has been in-substituted affinity enhancing nucleobase analogues (typically, this code will generate a complete list of possible chimeric sequences which are then examined for usefulness and at the same time removed from the 30 list in order to avoid double testing of the same chimeric sequence), f) executable code which can determine the usefulness of such chimeric sequences based on assessment of their ability to self-anneal and their melting temperatures and either rank such chimeric sequences according to their usefulness (this code is executed after execution of the code in step e, and basically functions as a iteration which tests each and every chimeric 35 sequence generated by feature e), g) storage means for storing at least one chimeric sequence (depending on the desired output, this storage means may hold a ranked list of chimeric sequences or one single

chimeric sequence, namely the one which has the highest degree of usefulness after each execution of one iteration in step f), and

h) output means for presenting the sequence of at least one optimized detection probe (will typically be a disk drive, a monitor or a printer).

5 Typically the target nucleic acid sequences stored in step b will be sequences of non-coding small RNAs as discussed herein.

Also a storage means embedding executable code (e.g. a computer program) which executes the design steps of the method referred to above is part of the present invention.

*Methods/uses of probes and probe collections*

10 Preferred methods/uses include: Specific isolation, purification, amplification, detection, identification, quantification, inhibition or capture of a target nucleotide sequence in a sample, by contacting said sample with a member of a collection of probes or a probe defined herein under conditions that facilitate hybridization between said member/probe and said target nucleotide sequence. Since the probes are typically shorter than the complete  
15 molecule wherein they form part, the inventive methods/uses include isolation, purification, amplification, detection, identification, quantification, inhibition or capture of a molecule comprising the target nucleotide sequence.

Typically, the molecule which is isolated, purified, amplified, detected, identified, quantified, inhibited or captured is a small, non-coding RNA, e.g. a miRNA such as a mature miRNA. A  
20 very surprising finding of the present invention is that it is possible to effect specific hybridization with miRNAs using probes of very short lengths, such as those lengths discussed herein when discussing the collection of probes. Typically the small, non-coding RNA has a length of at most 30 residues, such as at most 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, or 18 residues. The small non-coding RNA typically also has a length of at least 15  
25 residues, such as at least 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 residues.

As detailed in the examples herein, the specific hybridization between the short probes of the present invention to miRNA and the fact that miRNA can be mapped to various tissue origins, allows for an embodiment of the uses/methods of the present invention comprising  
30 identification of the primary site of metastatic tumors of unknown origin.

As also discussed in the examples herein, the short, but highly specific probes of the present invention allows hybridization assays to be performed on fixated embedded tissue sections,

such as formalin fixated paraffine embedded sections. Hence, an embodiment of the uses/methods of the present invention are those where the molecule, which is isolated, purified, amplified, detected, identified, quantified, inhibited or captured, is DNA (single stranded such as viral DNA) or RNA present in a fixated, embedded sample such as a  
5 formalin fixated paraffine embedded sample.

Other uses include:

- (a) capture and detection of naturally occurring or synthetic single stranded nucleic acids such as miRNAs, their target mRNAs, stem-loop precursor miRNAs, siRNAs, other non-coding RNAs, RNA-edited transcripts or alternative mRNA splice variants or viral DNA; or  
10 (b) purification of naturally occurring single stranded nucleic acids such as miRNAs, their target mRNAs, stem-loop precursor miRNAs, siRNAs, other non-coding RNAs, RNA-edited transcripts or alternative mRNA splice variants or viral DNA; or  
15 (c) detection and assessment of expression patterns for naturally occurring single stranded nucleic acids such as miRNAs, their target mRNAs, stem-loop precursor miRNAs, siRNAs, other non-coding RNAs, RNA-edited transcripts or alternative mRNA splice variants by RNA in-situ hybridisation, dot blot hybridisation, reverse dot blot hybridisation, or in Northern blot analysis or expression profiling by microarrays  
20 (d) functional analysis of naturally occurring single stranded nucleic acids such as miRNAs, their target mRNAs, stem-loop precursor miRNAs, siRNAs, other non-coding RNAs, RNA-edited transcripts or alternative mRNA splice variants or viral DNA in vitro and in vivo in plants or animals, such as human, mouse, rat, zebrafish, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, rice and maize, by inhibiting their mode of action, e.g. the binding of mature miRNAs to their cognate target mRNAs.  
25 (e) antisense-based intervention, targeted against tumorigenic single stranded nucleic acids such as miRNAs, their target mRNAs, stem-loop precursor miRNAs, siRNAs, other non-coding RNAs, RNA-edited transcripts or alternative mRNA splice variants or viral DNA in vivo in plants or animals, such as human, mouse, rat, zebrafish, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, rice and maize, by inhibiting their mode of action, e.g. the binding of mature miRNAs to their cognate target mRNAs.  
30 Further embodiments includes the use of an LNA modified oligonucleotide probe as an aptamer in molecular diagnostics or (b) as an aptamer in RNA mediated catalytic processes or (c) as an aptamer in specific binding of antibiotics, drugs, amino acids, peptides, structural

proteins, protein receptors, protein enzymes, saccharides, polysaccharides, biological cofactors, nucleic acids, or triphosphates or (d) as an aptamer in the separation of enantiomers from racemic mixtures by stereospecific binding or (e) for labelling cells or (f) to hybridise to non-protein coding cellular RNAs, such as tRNA, rRNA, snRNA and scRNA, in vivo or in-vitro or (g) to hybridise to non-protein coding cellular RNAs, such as tRNA, rRNA, snRNA and scRNA, in vivo or in-vitro or (h) in the construction of Taqman probes or Molecular Beacons.

The present invention also provides a kit for the isolation, purification, amplification, detection, identification, quantification, or capture of natural or synthetic nucleic acids, where 10 the kit comprises a reaction body and one or more LNAs as defined herein. The LNAs are preferably immobilised onto said reactions body (e.g. by using the immobilising techniques described above).

For the kits according to the invention, the reaction body is preferably a solid support material, e.g. selected from borosilicate glass, soda-lime glass, polystyrene, polycarbonate, 15 polypropylene, polyethylene, polyethyleneglycol terephthalate, polyvinylacetate, polyvinylpyrrolidinone, polymethylmethacrylate and polyvinylchloride, preferably polystyrene and polycarbonate. The reaction body may be in the form of a specimen tube, a vial, a slide, a sheet, a film, a bead, a pellet, a disc, a plate, a ring, a rod, a net, a filter, a tray, a microtitre plate, a stick, or a multi-bladed stick.

20 A written instruction sheet stating the optimal conditions for use of the kit typically accompanies the kits.

#### Further aspects of the invention

Once the appropriate target RNA sequences have been selected, LNA substituted detection probes are preferably chemically synthesized using commercially available methods and 25 equipment as described in the art (*Tetrahedron* 54: 3607-30, 1998). For example, the solid phase phosphoramidite method can be used to produce short LNA probes (Caruthers, et al., *Cold Spring Harbor Symp. Quant. Biol.* 47:411-418, 1982, Adams, et al., *J. Am. Chem. Soc.* 105: 661 (1983).

30 LNA-containing-probes can be labelled during synthesis. The flexibility of the phosphoramidite synthesis approach furthermore facilitates the easy production of LNAs carrying all commercially available linkers, fluorophores and labelling-molecules available for this standard chemistry. LNA-modified probes may also be labelled by enzymatic reactions e.g. by kinasing using T4 polynucleotide kinase and gamma-<sup>32</sup>P-ATP or by using terminal

deoxynucleotidyl transferase (TDT) and any given digoxigenin-conjugated nucleotide triphosphate (dNTP) or dideoxynucleotide triphosphate (ddNTP).

Detection probes according to the invention can comprise single labels or a plurality of labels. In one aspect, the plurality of labels comprise a pair of labels which interact with each other 5 either to produce a signal or to produce a change in a signal when hybridization of the detection probe to a target sequence occurs.

In another aspect, the detection probe comprises a fluorophore moiety and a quencher moiety, positioned in such a way that the hybridized state of the probe can be distinguished from the unhybridized state of the probe by an increase in the fluorescent signal from the 10 nucleotide. In one aspect, the detection probe comprises, in addition to the recognition element, first and second complementary sequences, which specifically hybridize to each other, when the probe is not hybridized to a recognition sequence in a target molecule, bringing the quencher molecule in sufficient proximity to said reporter molecule to quench 15 fluorescence of the reporter molecule. Hybridization of the target molecule distances the quencher from the reporter molecule and results in a signal, which is proportional to the amount of hybridization.

In the present context, the term "label" means a reporter group, which is detectable either by itself or as a part of a detection series. Examples of functional parts of reporter groups are biotin, digoxigenin, fluorescent groups (groups which are able to absorb electromagnetic 20 radiation, e.g. light or X-rays, of a certain wavelength, and which subsequently reemits the energy absorbed as radiation of longer wavelength; illustrative examples are DANSYL (5-dimethylamino)-1-naphthalenesulfonyl), DOXYL (N-oxyl-4,4-dimethyloxazolidine), PROXYL (N-oxyl-2,2,5,5-tetramethylpyrrolidine), TEMPO (N-oxyl-2,2,6,6-tetramethylpiperidine), dinitrophenyl, acridines, coumarins, Cy3 and Cy5 (trademarks for Biological Detection 25 Systems, Inc.), erythrosine, coumaric acid, umbelliferone, Texas red, rhodamine, tetramethyl rhodamine, Rox, 7-nitrobenzo-2-oxa-1-diazole (NBD), pyrene, fluorescein, Europium, Ruthenium, Samarium, and other rare earth metals), radio isotopic labels, chemiluminescence labels (labels that are detectable via the emission of light during a 30 chemical reaction), spin labels (a free radical (e.g. substituted organic nitroxides) or other paramagnetic probes (e.g. Cu<sup>2+</sup>, Mg<sup>2+</sup>) bound to a biological molecule being detectable by the use of electron spin resonance spectroscopy). Especially interesting examples are biotin, fluorescein, Texas Red, rhodamine, dinitrophenyl, digoxigenin, Ruthenium, Europium, Cy5, Cy3, etc.

Suitable samples of target nucleic acid molecules may comprise a wide range of eukaryotic 35 and prokaryotic cells, including protoplasts; or other biological materials, which may harbour

target nucleic acids. The methods are thus applicable to tissue culture animal cells, animal cells (e.g., blood, serum, plasma, reticulocytes, lymphocytes, urine, bone marrow tissue, cerebrospinal fluid or any product prepared from blood or lymph) or any type of tissue biopsy (e.g. a muscle biopsy, a liver biopsy, a kidney biopsy, a bladder biopsy, a bone biopsy, a 5 cartilage biopsy, a skin biopsy, a pancreas biopsy, a biopsy of the intestinal tract, a thymus biopsy, a mammae biopsy, a uterus biopsy, a testicular biopsy, an eye biopsy or a brain biopsy, e.g., homogenized in lysis buffer), archival tissue nucleic acids, plant cells or other cells sensitive to osmotic shock and cells of bacteria, yeasts, viruses, mycoplasmas, protozoa, rickettsia, fungi and other small microbial cells and the like.

10 Preferably, the detection probes of the invention are modified in order to increase the binding affinity of the probes for the target sequence by at least two-fold compared to probes of the same sequence without the modification, under the same conditions for hybridization or stringent hybridization conditions. The preferred modifications include, but are not limited to, inclusion of nucleobases, nucleosidic bases or nucleotides that have been modified by a 15 chemical moiety or replaced by an analogue to increase the binding affinity. The preferred modifications may also include attachment of duplex-stabilizing agents e.g., such as minor-groove-binders (MGB) or intercalating nucleic acids (INA). Additionally, the preferred modifications may also include addition of non-discriminatory bases e.g., such as 5-nitroindole, which are capable of stabilizing duplex formation regardless of the nucleobase at 20 the opposing position on the target strand. Finally, multi-probes composed of a non-sugar-phosphate backbone, e.g. such as PNA, that are capable of binding sequence specifically to a target sequence are also considered as a modification. All the different binding affinity-increasing modifications mentioned above will in the following be referred to as "the stabilizing modification(s)", and the tagging probes and the detection probes will in the 25 following also be referred to as "modified oligonucleotide". More preferably the binding affinity of the modified oligonucleotide is at least about 3-fold, 4-fold, 5-fold, or 20-fold higher than the binding of a probe of the same sequence but without the stabilizing modification(s).

30 Most preferably, the stabilizing modification(s) is inclusion of one or more LNA nucleotide analogs. Probes from 6 to 30 nucleotides according to the invention may comprise from 1 to 8 stabilizing nucleotides, such as LNA nucleotides. When at least two LNA nucleotides are included, these may be consecutive or separated by one or more non-LNA nucleotides. In one aspect, LNA nucleotides are alpha-L-LNA and/or xylo LNA nucleotides as disclosed in PCT Publications No. WO 2000/66604 and WO 2000/56748.

35 The problems with existing detection, quantification and knock-down of miRNAs and siRNAs as outlined above are addressed by the use of the novel oligonucleotide probes of the

invention in combination with any of the methods of the invention selected so as to recognize or detect a majority of all discovered and detected miRNAs, in a given cell type from a given organism. In one aspect, the probe sequences comprise probes that detect mammalian mature miRNAs, e.g., such as mouse, rat, rabbit, monkey, or human miRNAs. By providing a 5 sensitive and specific method for detection of mature miRNAs, the present invention overcomes the limitations discussed above especially for conventional miRNA assays and siRNA assays. The detection element of the detection probes according to the invention may be single or double labelled (e.g. by comprising a label at each end of the probe, or an 10 internal position). In one aspect, the detection probe comprises two labels capable of interacting with each other to produce a signal or to modify a signal, such that a signal or a change in a signal may be detected when the probe hybridizes to a target sequence. A particular aspect is when the two labels comprise a quencher and a reporter molecule.

In another aspect, the probe comprises a target-specific recognition segment capable of 15 specifically hybridizing to a target molecule comprising the complementary recognition sequence. A particular detection aspect of the invention referred to as a "molecular beacon with a stem region" is when the recognition segment is flanked by first and second complementary hairpin-forming sequences which may anneal to form a hairpin. A reporter label is attached to the end of one complementary sequence and a quenching moiety is attached to the end of the other complementary sequence. The stem formed when the first 20 and second complementary sequences are hybridized (i.e., when the probe recognition segment is not hybridized to its target) keeps these two labels in close proximity to each other, causing a signal produced by the reporter to be quenched by fluorescence resonance energy transfer (FRET). The proximity of the two labels is reduced when the probe is hybridized to a target sequence and the change in proximity produces a change in the 25 interaction between the labels. Hybridization of the probe thus results in a signal (e.g. fluorescence) being produced by the reporter molecule, which can be detected and/or quantified.

As mentioned above, the invention also provides a method, system and computer program 30 embedded in a computer readable medium ("a computer program product") for designing detection probes comprising at least one stabilizing nucleobase. The method comprises querying a database of target sequences (e.g., such as the miRNA registry at 35 <http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml>) and designing probes which: i) have sufficient binding stability to bind their respective target sequence under stringent hybridization conditions, ii) have limited propensity to form duplex structures with itself, and iii) are capable of binding to and detecting/quantifying at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 95% of all the target sequences in the given database of miRNAs or other RNA sequences.

In one preferred aspect, the target sequence database comprises nucleic acid sequences corresponding to human, mouse, rat, *Drosophila melanogaster*, *c. elegans*, *Arabidopsis thaliana*, maize or rice miRNAs.

In another aspect, the method further comprises calculating stability based on the

5 assumption that the recognition sequence comprises at least one stabilizing nucleotide, such as an LNA molecule. In one preferred aspect the calculated stability is used to eliminate probes with inadequate stability from the database of virtual candidate probes prior to the initial query against the database of target sequence to initiate the identification of optimal probe recognition sequences.

10 In another aspect, the method further comprises calculating the capability for a given probe sequence to form a duplex structure with itself based on the assumption that the sequence comprises at least one stabilizing nucleotide, such as an LNA molecule. In one preferred aspect the calculated propensity is used to eliminate probe sequences that are likely to form probe duplexes from the database of virtual candidate probes.

15 A preferred embodiment of the invention are kits for the detection or quantification of target miRNAs, siRNAs, RNA-edited transcripts, non-coding antisense transcripts or alternative splice variants comprising libraries of detection probes. In one aspect, the kit comprises *in silico* protocols for their use. The detection probes contained within these kits may have any or all of the characteristics described above. In one preferred aspect, a plurality of probes

20 comprises at least one stabilizing nucleotide, such as an LNA nucleotide. In another aspect, the plurality of probes comprises a nucleotide coupled to or stably associated with at least one chemical moiety for increasing the stability of binding of the probe. The kits according to the invention allow a user to quickly and efficiently develop an assay for different miRNA targets, siRNA targets, RNA-edited transcripts, non-coding antisense transcripts or alternative

25 splice variants.

The invention also provides a method, system and computer program embedded in a computer readable medium ("a computer program product") for designing detection probes comprising at least one stabilizing nucleobase. The method comprises querying a database of target sequences (e.g., such as the miRNA registry at

30 <http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml> ) and designing probes which: i) have sufficient binding stability to bind their respective target sequence under stringent hybridization conditions, ii) have limited propensity to form duplex structures with itself, and iii) are capable of binding to and detecting/quantifying at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 95% of all the target

35 sequences in the given database of miRNAs or other RNA sequences.

In one preferred aspect, the target sequence database comprises nucleic acid sequences corresponding to human, mouse, rat, *Drosophila melanogaster*, *C. elegans*, *Arabidopsis thaliana*, maize or rice miRNAs.

1 In another aspect, the method further comprises calculating stability based on the  
5 assumption that the recognition sequence comprises at least one stabilizing nucleotide, such as an LNA molecule. In one preferred aspect the calculated stability is used to eliminate probes with inadequate stability from the database of virtual candidate probes prior to the initial query against the database of target sequence to initiate the identification of optimal probe recognition sequences.

10 In another aspect, the method further comprises calculating the capability for a given probe sequence to form a duplex structure with itself based on the assumption that the sequence comprises at least one stabilizing nucleotide, such as an LNA molecule. In one preferred aspect the calculated propensity is used to eliminate probe sequences that are likely to form probe duplexes from the database of virtual candidate probes.

15 In general, the invention features the design of high affinity oligonucleotide probes that have duplex stabilizing properties and methods highly useful for a variety of target nucleic acid detection methods (e.g., monitoring spatiotemporal expression of microRNAs or siRNAs or knock-down of miRNAs). Some of these oligonucleotide probes contain novel nucleotides created by combining specialized synthetic nucleobases with an LNA backbone, thus creating  
20 high affinity oligonucleotides with specialized properties such as reduced sequence discrimination for the complementary strand or reduced ability to form intramolecular double stranded structures. The invention also provides improved methods for detecting and quantifying ribonucleic acids in complex nucleic acid sample. Other desirable modified bases have decreased ability to self-anneal or to form duplexes with oligonucleotide probes  
25 containing one or more modified bases.

#### EXAMPLES

The invention will now be further illustrated with reference to the following examples. It will be appreciated that what follows is by way of example only and that modifications to detail may be made while still falling within the scope of the invention.

## EXAMPLE 1

*Synthesis, deprotection and purification of LNA-substituted oligonucleotide probes*

The LNA-substituted probes of Example 2 to 11 were prepared on an automated DNA synthesizer (Expedite 8909 DNA synthesizer, PerSeptive Biosystems, 0.2  $\mu$ mol scale) using 5 the phosphoramidite approach (Beaucage and Caruthers, *Tetrahedron Lett.* 22: 1859-1862, 1981) with 2-cyanoethyl protected LNA and DNA phosphoramidites, (Sinha, et al., *Tetrahedron Lett.* 24: 5843-5846, 1983). CPG solid supports derivatised with a suitable quencher and 5'-fluorescein phosphoramidite (GLEN Research, Sterling, Virginia, USA). The synthesis cycle was modified for LNA phosphoramidites (250s coupling time) compared to 10 DNA phosphoramidites. 1H-tetrazole or 4,5-dicyanoimidazole (Proligo, Hamburg, Germany) was used as activator in the coupling step.

The probes were deprotected using 32% aqueous ammonia (1h at room temperature, then 2 hours at 60°C) and purified by HPLC (Shimadzu-SpectraChrom series; Xterra<sup>TM</sup> RP18 column, 10 $\mu$ m 7.8 x 150 mm (Waters). Buffers: A: 0.05M Triethylammonium acetate pH 7.4. B. 50% 15 acetonitrile in water. Eluent: 0-25 min: 10-80% B; 25-30 min: 80% B). The composition and purity of the probes were verified by MALDI-MS (PerSeptive Biosystem, Voyager DE-PRO) analysis.

## EXAMPLE 2

*List of LNA-substituted detection probes for detection of fully conserved vertebrate 20 microRNAs in all vertebrates*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze 25 conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard 30 methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

TABLE A

<b>LNA probe name</b>	<b>Sequence 5'-3'</b>	<b>Self-comp score</b>	<b>Calculated Tm</b>
hsa-let7f/LNA	aamCtaTacAatmCtamCtamCctmCa	16	67
hsa-miR19b/LNA	tmCagTttTgcAtgGatTtgmCaca	34	75
hsa-miR17-5p/LNA	actAccTgcActGtaAgcActTtg	39	74
hsa-miR217/LNA	atcmCaaTcaGttmCctGatGcaGta	49	75
hsa-miR218/LNA	acAtgGttAgaTcaAgcAcaa	40	70
hsa-miR222/LNA	gaGacmCcaGtaGccAgaTgtAgct	38	80
hsa-let7i/LNA	agmCacAaamCtamCtamCctmCa	18	71
hsa-miR27b/LNA	cagAacTtaGccActGtgAa	35	68
hsa-miR301/LNA	gctTtgAcaAtamCtaTtgmCacTg	36	70
hsa-miR30b/LNA	gcTgaGtgTagGatGttTaca	33	70
hsa-miR100/LNA	cacAagTtcGgaTctAcgGtt	38	77
hsa-miR34a/LNA	aamCaamCcaGctAagAcamCtgmCca	27	80
hsa-miR7/LNA	aacAaaAtcActAgtmCttmCca	30	66
hsa-miR125b/LNA	tcamCaaGttAggGtcTcaGgga	35	77
hsa-miR133a/LNA	acAgcTggTtgAagGggAccAa	41	82
hsa-miR101/LNA	cttmCagTtaTcamCagTacTgta	54	68
hsa-miR108/LNA	aatGccmCctAaaAatmCctTat	23	66
hsa-miR107/LNA	tGatAgcmCctGtamCaaTgcTgct	63	80
hsa-miR153/LNA	tcamCttTtgTgamCtaTgcAa	35	68
hsa-miR10b/LNA	amCaaAttmCggTtcTacAggGta	35	73
mmu-miR10b/LNA	acamCaaAttmCggTtcTacAggg	27	73
hsa-miR194/LNA	tccAcaTggAgtTgcTgtTaca	41	75
hsa-miR199a/LNA	gaAcaGgtAgtmCtgAacActGgg	40	78
hsa-miR199a*/LNA	aacmCaaTgtGcaGacTacTgta	39	74
hsa-miR20/LNA	ctAccTgcActAtaAgcActTta	26	70
hsa-miR214/LNA	ctGccTgtmCtgTgcmCtgmCtgt	30	81
hsa-miR219/LNA	agAatTgcGttTggAcaAtca	35	70
hsa-miR223/LNA	gGggTatTtgAcaAacTgamCa	40	73
hsa-miR23a/LNA	gGaaAtcmCctGgcAatGtgAt	37	76
hsa-miR24/LNA	cTgtTccTgcTgaActGagmCca	35	80
hsa-miR26a/LNA	agcmCtaTccTggAttActTgaa	34	70
hsa-miR126/LNA	gcAttAttActmCacGgtAcga	25	71
hsa-miR126*/LNA	cgmCgtAccAaaAgtAatAatg	28	68

<b>LNA probe name</b>	<b>Sequence 5'-3'</b>	<b>Self-comp score</b>	<b>Calculated Tm</b>
hsa-miR128a/LNA	aaAagAgamCcgGtmtCacTgtGa	47	77
mmu-miR7b/LNA	aamCaaAatmCacAagTctTcca	24	68
hsa-let7c/LNA	aamCcaTacAacmCtamCtamCctmCa	11	74
hsa-let7b/LNA	aamCcamCacAacmCtamCtamCctmCa	6	77
hsa-miR103/LNA	tmCatAgcmCctGtamCaaTgcTgct	63	80
hsa-miR129/LNA	agcAagmCccAgamCggmCaaAaag	21	80
rno-miR129*/LNA	aTgcTttTtgGggTaaGggmCtt	37	78
hsa-miR130a/LNA	gcmCctTttAacAttGcamCtg	34	70
hsa-miR132/LNA	cgAccAtgGctGtaGacTgtTa	48	76
hsa-miR135a/LNA	tcamCatAggAatAaaAagmCcaTa	22	69
hsa-miR137/LNA	cTacGcgTatTctTaaGcaAta	48	68
hsa-miR200a/LNA	acaTcgTtamCcaGacAgtGtta	39	72
hsa-miR142-3p/LNA	tmCcaTaaAgtAggAaamCacTaca	29	72
hsa-miR142-5p/LNA	gtaGtgmCttTctActTtaTg	36	63
hsa-miR181b/LNA	aamCccAccGacAgcAatGaaTgtt	30	81
hsa-miR183/LNA	caGtgAatTctAccAgtGccAta	32	73
hsa-miR190/LNA	acmCtaAtaTatmCaaAcaTatmCa	31	62
hsa-miR193/LNA	ctGggActTtgTagGccAgtt	31	76
hsa-miR19a/LNA	tmCagTttTgcAtaGatTtgmCaca	37	72
hsa-miR204/LNA	cagGcaTagGatGacAaaGggAa	25	78
hsa-miR205/LNA	caGacTccGgtGgaAtgAagGa	39	81
hsa-miR216/LNA	camCagTtgmCcaGctGagAtta	64	74
hsa-miR221/LNA	gAaamCccAgcAgamCaaTgtAgct	31	80
hsa-miR25/LNA	tcaGacmCgaGacAagTgcAatg	27	77
hsa-miR29c/LNA	taamCcgAttTcaAatGgtGcta	47	70
hsa-miR29b/LNA	amCacTgaTttmCaaAtgGtgmCta	47	71
hsa-miR30c/LNA	gmCtgAgaGtgTagGatGttTaca	33	73
hsa-miR140/LNA	ctAccAtaGggTaaAacmCact	43	71
hsa-miR9*/LNA	acTttmCggTtaTctAgtTta	27	65
hsa-miR92/LNA	amCagGccGggAcaAgtGcaAta	36	81
hsa-miR96/LNA	aGcaAaaAtgTgcTagTgcmCaaa	38	75
hsa-miR99a/LNA	cacAagAtcGgaTctAcgGgtt	42	77
hsa-miR145/LNA	aAggGatTccTggGaaAacTggAc	50	79
hsa-miR155/LNA	ccmCctAtcAcgAttAgtGcaAta	29	71
hsa-miR29a/LNA	aamCcgAttTcaAatGgtGctAg	47	75

LNA probe name	Sequence 5'-3'	Self-comp score	Calculated Tm
rno-miR140*/LNA	gtcmCgtGgtTctAccmCtgTggTa	49	81
hsa-miR206/LNA	ccamCacActTccTtamCatTcca	11	73
hsa-miR124a/LNA	tggmCatTcamCcgmCgtGccTtaa	43	80
hsa-miR122a/LNA	acAaamCacmCatTgtmCacActmCca	25	78
hsa-miR1/LNA	tamCatActTctTtamCatTcca	11	64
hsa-miR181a/LNA	acTcamCcgAcaGcgTtgAatGtt	49	77
hsa-miR10a/LNA	cAcaAatTcgGatmCtamCagGgta	37	74
hsa-miR196a/LNA	ccaAcaAcaTgaAacTacmCta	20	67
hsa-let7a/LNA	aamCtaTacAacmCtamCtamCctmCa	16	70
hsa-miR9/LNA	tcAtamCagmCtaGatAacmCaaAga	34	71

## EXAMPLE 3

*List of LNA-substituted detection probes for detection of fully conserved vertebrate microRNAs in all vertebrates*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

## 15 TABLE B

Probe name	Sequence 5'-3'	Self-compl score	Calculated Tm
hsa-miR-210	agcmCgcTgtmCacAcgmCacAg	37	84
hsa-miR-144	taGtamCatmCatmCtaTacTgta	37	64
hsa-miR-338	caAcaAaaTcamCtgAtgmCtgGa	33	72

Probe name	Sequence 5'-3'	Self-compl score	Calculated Tm
hsa-miR-187	ggcTgcAacAcaAgamCacGa	30	79
hsa-miR-200b	cAtcAttAccAggmCagTatTaga	29	71
hsa-miR-184	cmCctTatmCagTtcTccGtcmCa	23	75
hsa-miR-27a	gcGgaActTagmCcamCtgTgaa	35	77
hsa-miR-215	ctgTcaAttmCatAggTcat	38	65
hsa-miR-203	agTggTccTaaAcaTttmCac	23	68
hsa-miR-16	ccaAtaTttAcgTgcTgcTa	30	68
hsa-miR-152	aAgtTctGtcAtgmCacTga	29	72

## EXAMPLE 4

*List of LNA-substituted detection probes for detection of zebrafish microRNAs*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze 5 conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe 10 sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH2-C6- or a NH2-C6-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

TABLE C

Probe name	Sequence 5'-3'	Self-comp score	Calculated Tm
dre-miR-93	ctAccTgcAcaAacAgcActTt	26	73
dre-miR-22	acaGttmCttmCagmCtgGcaGctt	62	76
dre-miR-213	gGtamCagTcaAcgGtcGatGgt	63	80
dre-miR-31	cagmCtaTgcmCaamCatmCttGcc	34	76
dre-miR-189	amCtgTtaTcaGctmCagTagGcac	41	75
dre-miR-18	tatmCtgmCacTaaAtgmCacmCtta	45	69

dre-miR-15a	cAcaAacmCatTctGtgmCtgmCta	35	74
dre-miR-34b	cAatmCagmCtaAcaAcamCtgmCcta	24	74
dre-miR-148a	acaAagTtcTgtAatGcamCtga	44	69
dre-miR-125a	camCagGttAagGgtmCtcAggGa	38	80
dre-miR-139	agAcamCatGcamCtgTaga	34	69
dre-miR-150	cacTggTacAagGatTggGaga	30	75
dre-miR-192	ggcTgtmCaaTtcAtaGgtmCa	46	73
dre-miR-98	aacAacAcaActTacTacmCtca	17	68
dre-let-7g	amCtgTacAaamCaamCtamCctmCa	30	73
dre-miR-30a-5p	gctTccAgtmCggGgaTgtTtamCa	45	80
dre-miR-26b	aacmCtaTccTggAttActTgaa	36	68
dre-miR-21	cAacAccAgtmCtgAtaAgcTa	35	72
dre-miR-146	accmCttGgaAttmCagTtcTca	40	72
dre-miR-182	tgtGagTtcTacmCatTgcmCaaa	32	72
dre-miR-182*	taGttGgcAagTctAgaAcca	32	72
dre-miR-220	aAgtGtcmCgaTacGgtTgtGg	47	81

## EXAMPLE 5

*List of LNA-substituted detection probes for detection of *Drosophila melanogaster* microRNAs.*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze

5 conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe 10 sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH2-C6- or a NH2-C6-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

TABLE D

Probe name	Sequence 5'-3'	Self-compl score	Calculated Tm
dme-miR-2c	gcmCcaTcaAagmCtgGctGtgAta	68	78
dme-miR-6	aaaAagAacAgcmCacTgtGata	36	71
dme-miR-7	amCaamCaaAatmCacTagTctTcca	30	71
dme-miR-14	tAggAgaGagAaaAagActGa	15	71
dme-miR-277	tgTcgTacmCagAtaGtgmCatTta	38	72
dme-miR-278	aaAcgGacGaaAgtmCccAccGa	41	80
dme-miR-279	tTaaTgaGtgTggAtcTagTca	40	70
dme-miR-309	tAggAcaAacTttAccmCagTgc	37	74
dme-miR-310	aAagGccGggAagTgtGcaAta	28	79
dme-miR-318	tgaGatAaamCaaAgcmCcaGtga	25	73
dme-miR-bantam	aaTcaGctTtcAaaAtgAtcTca	40	66

## EXAMPLE 6

*List of LNA-substituted detection probes for detection of *Drosophila melanogaster* and *Caenorhabditis elegans* microRNAs*

5 LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

10

15

TABLE E

Probe name	Sequence 5'-3'	Self-comp score	Calculated Tm
dme_cel-miR1/LNA	cAtamCttmCttTacAttmCca	14	62
dme_cel-miR2/LNA	tcaAagmCtgGctGtgAta	56	67
cel-lin4/LNA	tcAcamCttGagGtcTcag	50	68

EXAMPLE 7

*List of LNA-substituted detection probes for detection of *Arabidopsis thaliana* micro RNAs*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

15 TABLE F

Probe name	Sequence 5'-3'	Self-comp score	Calculated Tm
ath-MIR171_LNA2	gAtAtTgGcGcGgmCtmCaAtmCa	64	83
ath-MIR171_LNA3	gAtaTtgGcgmCggmCtcAatmCa	54	78
ath-MIR159_LNA2	tAgAgmCtmCcmCtTcAaTcmCaAa	46	79
ath-MIR159_LNA3	tAgaGctmCccTtcAatmCcaAa	43	72
ath-MIR161LNA3	cmCccGatGtaGtcActTtcAa	34	73
ath-MIR167LNA3	tAgaTcaTgcTggmCagmCttmCa	53	79
ath-MIR319LNA3	ggGagmCtcmCctTcaGtcmCaa	70	78

## EXAMPLE 8

*List of LNA-substituted detection probes for detection of *Arabidopsis thaliana* microRNAs*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze

5 conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe  
10 sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

TABLE G

Oligo name	Sequence 5'-3'	Predicted Tm °C
ath-miR159a/LNA	tAgaGctmCccTtcAatmCcaAa	145
ath-miR319a/LNA	ggGagmCtcmCctTcaGtcmCaa	183
ath-miR396a/LNA	gTtcAagAaaGctGtgGaa	242
ath-miR156a/LNA	gtgmCtcActmCtcTtcTgtmCa	235
ath-miR172a/LNA	atgmCagmCatmCatmCaaGatTct	228

## 15 EXAMPLE 9

*List of LNA-substituted detection probes useful as controls in detection of vertebrate microRNAs*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze

20 conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe  
25 sequences can be used as capture sequences for expression profiling by LNA oligonucleotide

microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

TABLE H

Probe name	Sequence 5'-3'	Self-comp score
hsa-miR206/LNA/2MM	ccamCacActmCtcTtamCatTcca	8
hsa-miR206/LNA/MM10	ccamCacActmCccTtamCatTcca	8
hsa-miR124a/LNA/2MM	tggmCatTcaAagmCgtGccTtaa	60
hsa-miR124a/LNA/MM10	tggmCatTcaAcgmCgtGccTtaa	60
hsa-miR122a/LNA/2MM	acAaamCacmCacmCgtmCacActmCca	18
hsa-miR122a/LNA/MM11	acAaamCacmCatmCgtmCacActmCca	18

## 5 EXAMPLE 10

*List of LNA-substituted detection probes for detection of human microRNAs*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine, PM perfect match to the miRNA, MM one mismatch at the central position of the probe sequence. The detection probes can be used to detect and analyze conserved vertebrate miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the probes as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods. 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

TABLE I

Probe name	Sequence 5'-3'
hsa-let7a/LNA_PM	aamCtaTacAacmCtamCtamCctmCa
hsa-let7f/LNA_PM	aamCtaTacAatmCtamCtamCctmCa

Probe name	Sequence 5'-3'
hsa-miR143LNA_PM	tGagmCtamCagTgcTtcAtcTca
hsa-miR145/LNA_PM	aAggGatTccTggGaaAacTggAc
hsa-miR320/LNA_PM	tTcgmCccTctmCaamCccAgcTttt
hsa-miR26a/LNA_PM	agcmCtaTccTggAttActTgaa
hsa-miR99a/LNA_PM	cacAagAtcGgaTctAcgGgtt
hsa-miR15a/LNA_PM	cAcaAacmCatTatGtgmCtgmCta
hsa-miR16-1/LNA_PM	cgmCcaAtaTttAcgTgcTgcTa
hsa-miR24/LNA_PM	cTgtTccTgcTgaActGagmCca
hsa-let7g/LNA_PM	amCtgTacAaamCtamCtamCctmCa
hsa-let7a/LNA_MM	aamCtaTacAacAtamCtamCctmCa
hsa-let7f/LNA_MM	aamCtaTacAatAtamCtamCctmCa
hsa-miR143LNA_MM	tGagmCtamCagmCgcTtcAtcTca
hsa-miR145/LNA_MM	aAggGatTccTcgGaaAacTggAc
hsa-miR320/LNA_MM	tTcgmCccTctAaamCccAgcTttt
hsa-miR26a/LNA_MM	agcmCtaTccTcgAttActTgaa
hsa-miR99a/LNA_MM	cacAagAtcGcaTctAcgGgtt
hsa-miR15a/LNA_MM	cAcaAacmCatmCatGtgmCtgmCta
hsa-miR16-1/LNA_MM	cgmCcaAtaTttTcgTgcTgcTa
hsa-miR24/LNA_MM	cTgtTccTgcmCgaActGagmCca
hsa-let7g/LNA_MM	amCtgTacAaaAtamCtamCctmCa

## EXAMPLE 11

*List of LNA-substituted detection probes for expression profiling of human and mouse microRNAs by oligonucleotide microarrays*

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC

5 denotes LNA methyl-cytosine, PM perfect match to the miRNA, MM one mismatch at the central position of the probe sequence, dir denotes the probe sequence corresponding to the mature miRNA sequence, rev denotes the probe sequence complementary to the mature miRNA sequence in question. The detection probes can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid 10 surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer group at the 5'-end or at the 3'-end of the probes during synthesis.

TABLE 1

Probe name	Sequence 5'-3'	Self-comp score
mmu~let7adirPM/LNA	tgaGgtAgtAggTtgTatAgtt	30
mmu-miRldirPM/LNA	tgGaaTgtAaaGaaGtaTgta	18
mmu-miR16dirPM/LNA	tagmCagmCacGtaAatAttGgcg	46
mmu-miR22dirPM/LNA	aagmCtgmCcaGttGaaGaamCtgt	48
mmu-miR26bdirPM/LNA	tTcaAgtAatTcaGgaTagGtt	35
mmu-miR30cdirPM/LNA	tgtAaamCatmCctAcamCtcTcaGc	27
mmu-miR122adirPM/LNA	tggAgtGtgAcaAtgGtgTttg	32
mmu-miR126stardirPM/LNA	catTatTacTttTggTacGcg	28
mmu-miR126dirPM/LNA	tcgTacmCgtGagTaaTaaTgc	32
mmu-miR133dirPM/LNA	tTggTccmCctTcaAccAgcTgt	37
mmu-miR143dirPM/LNA	tGagAtgAagmCacTgtAgtTca	49
mmu-miR144dirPM/LNA	tAcaGtaTagAtgAtgTacTag	41
mmu-let7arevPM/LNA	aamCtaTacAacmCtamCtamCctmCa	16
mmu-miRlrevPM/LNA	tamCatActTctTtamCatTcca	11
mmu-miR16revPM/LNA	cgmCcaAtaTttAcgTgcTgcTa	34
mmu-miR22revPM/LNA	acaGttmCttmCaamCtgGcaGctt	48
mmu-miR26brevPM/LNA	aacmCtaTccTgaAttActTgaa	28
mmu-miR30crevPM/LNA	gmCtgAgaGtgTagGatGttTaca	33
mmu-miR122arevPM/LNA	cAaamCacmCatTgtmCacActmCca	25
mmu-miR126starrevPM/LNA	cgmCgtAccAaaAgtAatAatg	28
mmu-miR126revPM/LNA	gcAttAttActmCacGgtAcga	25
mmu-miR133revPM/LNA	acAgtTggTtgAagGggAccAa	41
mmu-miR143revPM/LNA	tGagmCtamCagTgcTtcAtcTca	56
mmu-miR144revPM/LNA	ctaGtamCatmCatmCtaTacTgta	37
mmu-let7adirMM/LNA	tgaGgtAgtAagTtgTatAgtt	34
mmu-miRldirMM/LNA	tgGaaTgtAagGaaGtaTgta	18
mmu-miR16dirMM/LNA	tAgcAgcAcgGaaAtaTtgGcg	33
mmu-miR22dirMM/LNA	aaGctGccAggTgaAgaActGt	35
mmu-miR26bdirl YM/LNA	tTcaAgtAatGcaGgaTagGtt	27
mmu-miR30cdirMM/LNA	tgtAaamCatmCatAcamCtcTcaGc	27
mmu-miR122adirMM/LNA	tggAgtGtgAaaAtgGtgTttg	29
mmu-miR126stardirMM/LNA	catTatTacTgtTggTacGcg	35
mmu~miR126dirMM/LNA	tmCgtAccGtgGgtAatAatGc	39
mmu-miR133dirMM/LNA	ttgGtcmCccTgcAacmCagmCtgt	42

Probe name	Sequence 5'-3'	Self-comp score
mmu-miR143dirMM/LNA	tGagAtgAagAacTgtAgcTca	49
mmu-miR144dirMM/LNA	tAcaGtaTagGtgAtgTacTag	41
mmu-let7arevMM/LNA	aActAtamCaamCttActAccTca	17
mmu-miR1revMM/LNA	tacAtamCttmCctTacAttmCca	11
mmu-miR16revMM/LNA	cgmCcaAtaTttmCcgTgcTgcTa	34
mmu-miR22revMM/LNA	amCagTtcTtcAccTggmCagmCtt	35
mmu-miR26brevMM/LNA	aamCctAtcmCtgmCatTacTtgAa	24
mmu-miR30crevMM/LNA	gmCtgAgaGtgTatGatGttTaca	29
mmu-miR122arevMM/LNA	cAaamCacmCatTttmCacActmCca	13
mmu-miR126starrevMM/LNA	cgmCgtAccAacAgtAatAatg	31
mmu-miR126revMM/LNA	gmCatTatTacmCcamCggTacGa	39
mmu-miR133revMM/LNA	acaGctGgtTgcAggGgamCcaa	45
mmu-miR143revMM/LNA	tgAgtTacAgtTctTcaTctmCa	49
mmu-miR144revMM/LNA	ctAgtAcaTcamCctAtamCtgTa	31

## EXAMPLE 12

*List of LNA-substituted detection probes for detection of all microRNAs listed in the miRNA registry database release 5.1 from December 2004 at*  
<http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml>

5 LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine. The detection probes can be used to detect and analyze miRNAs by RNA in situ hybridization, Northern blot analysis and by silencing using the oligonucleotides as miRNA inhibitors. The LNA-modified probes can be conjugated with a variety of haptens or fluorochromes for miRNA in situ hybridization using standard methods.

10 5'-end labeling using T4 polynucleotide kinase and gamma-32P-ATP can be carried out by standard methods for Northern blot analysis. In addition, the LNA-modified probe sequences can be used as capture sequences for expression profiling by LNA oligonucleotide microarrays. Covalent attachment to the solid surfaces of the capture probes can be accomplished by incorporating a NH<sub>2</sub>-C<sub>6</sub>- or a NH<sub>2</sub>-C<sub>6</sub>-hexaethylene glycol monomer or dimer

15 group, or a NH<sub>2</sub>-C<sub>3</sub>-random N<sub>2</sub>O sequence at the 5'-end or at the 3'-end of the probes during synthesis. Ath, *Arabidopsis thaliana*; cbr, *Caenorhabditis briggsae*; eel, *Caenorhabditis elegans*; dme, *Drosophila melanogaster*, dps, *Drosophila pseudoobscura*; dre, *Danio rerio*; ebr, Eppstein Barr Virus; gga, *Gallus gallus*; has, *Homo sapiens*; mmu, *Mus musculus*; osa, *Oryza sativa*; rno, *Rattus norvegicus*; zma, *Zea mays*.

TABLE K

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
ath-miR156a	gtgmCtcActmCtcTtcTgtmCa	71	25
ath-miR156b	gtgmCtcActmCtcTtcTgtmCa	71	25
ath-miR156c	gtgmCtcActmCtcTtcTgtmCa	71	25
ath-miR156d	gtgmCtcActmCtcTtcTgtmCa	71	25
ath-miR156e	gtgmCtcActmCtcTtcTgtmCa	71	25
ath-miR156f	gtgmCtcActmCtcTtcTgtmCa	71	25
ath-miR156g	tgTgcTcamCtcTctTctGtcg	74	31
ath-miR156h	gtgmCtcTctTtcTtcTgtmCaa	68	25
ath-miR157a	gtgmCtcTctAtcTtcTgtmCaa	68	25
ath-miR157b	gtgmCtcTctAtcTtcTgtmCaa	68	25
ath-miR157c	gtgmCtcTctAtcTtcTgtmCaa	68	25
ath-miR157d	gTgcTctmCtaTctTctGtca	69	21
ath-miR158a	tgmCttTgtmCtamCatTtgGga	71	28
ath-miR158b	tgmCttTgtmCtamCatTtgGgg	72	28
ath-miR159a	tagAgcTccmCttmCaaTccAaa	71	36
ath-miR159b	aagAgcTccmCttmCaaTccAaa	72	36
ath-miR159c	aggAgcTccmCttmCaaTccAaa	74	46
ath-miR160a	tggmCatAcaGggAgcmCagGca	85	49
ath-miR160b	tggmCatAcaGggAgcmCagGca	85	49
ath-miR160c	tggmCatAcaGggAgcmCagGca	85	49
ath-miR161	cccmCgaTgtAgtmCacTttmCaa	75	27
ath-miR162a	ctgGatGcaGagGttTatmCga	73	34
ath-miR162b	ctgGatGcaGagGttTatmCga	73	34
ath-miR163	aTcgAagTtcmCaaGtcmCtcTtcAa	74	29
ath-miR164a	tgcAcgTgcmCctGctTctmCca	82	46
ath-miR164b	tgcAcgTgcmCctGctTctmCca	82	46
ath-miR164c	cgcAcgTgcmCctGctTctmCca	83	46
ath-miR165a	gggGgaTgaAgcmCtgGtcmCga	84	46
ath-miR165b	gggGgaTgaAgcmCtgGtcmCga	84	46
ath-miR166a	gGggAatGaaGccTggTccGa	84	33
ath-miR166b	gGggAatGaaGccTggTccGa	84	33
ath-miR166c	gGggAatGaaGccTggTccGa	84	33
ath-miR166d	gGggAatGaaGccTggTccGa	84	33

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
ath-miR166e	gGggAatGaaGccTggTccGa	84	33
ath-miR166f	gGggAatGaaGccTggTccGa	84	33
ath-miR166g	gGggAatGaaGccTggTccGa	84	33
ath-miR167a	tAgaTcaTgcTggmCagmCttmCa	79	53
ath-miR167b	tAgaTcaTgcTggmCagmCttmCa	79	53
ath-miR167c	aAgaTcaTgcTggmCagmCttAa	76	53
ath-miR167d	ccAgaTcaTgcTggmCagmCttmCa	82	53
ath-miR168a	ttcmCcgAccTgcAccAagmCga	82	26
ath-miR168b	ttcmCcgAccTgcAccAagmCga	82	26
ath-miR169a	tcGgcAagTcaTccTtgGctg	78	40
ath-miR169b	ccGgcAagTcaTccTtgGctg	79	40
ath-miR169c	ccGgcAagTcaTccTtgGctg	79	40
ath-miR169d	cGgcAagTcaTccTtgGctmCa	80	35
ath-miR169e	cGgcAagTcaTccTtgGctmCa	80	35
ath-miR169f	cGgcAagTcaTccTtgGctmCa	80	35
ath-miR169g	cGgcAagTcaTccTtgGctmCa	80	35
ath-miR169g*	aGccAagGtcAacTtgmCcgGa	81	45
ath-miR169h	caGgcAagTcaTccTtgGcta	76	41
ath-miR169i	caGgcAagTcaTccTtgGcta	76	41
ath-miR169j	caGgcAagTcaTccTtgGcta	76	41
ath-miR169k	caGgcAagTcaTccTtgGcta	76	41
ath-miR169l	caGgcAagTcaTccTtgGcta	76	41
ath-miR169m	caGgcAagTcaTccTtgGcta	76	41
ath-miR169n	caGgcAagTcaTccTtgGcta	76	41
ath-miR170	gAtaTtgAcamCggmCtcAatmCa	72	52
ath-miR171a	gAtaTtgGcgmCggmCtcAatmCa	78	54
ath-miR171b	cGtgAtaTtgGcamCggmCtcAa	77	43
ath-miR171c	cGtgAtaTtgGcamCggmCtcAa	77	43
ath-miR172a	atgmCagmCatmCatmCaaGatTct	73	45
ath-miR172b	atgmCagmCatmCatmCaaGatTct	73	45
ath-miR172b*	gtgAatmCttAatGgtGctGc	72	33
ath-miR172c	ctgmCagmCatmCatmCaaGatTct	73	39
ath-miR172d	ctgmCagmCatmCatmCaaGatTct	73	39
ath-miR172e	aTgcAgcAtcAtcAagAttmCc	74	39
ath-miR173	gtgAttTctmCtcTgcAagmCgaa	72	38

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
ath-miR319a	gggAgcTccmCttmCagTccAa	77	64
ath-miR319b	gggAgcTccmCttmCagTccAa	77	64
ath-miR319c	aggAgcTccmCttmCagTccAa	76	46
ath-miR393a	gAtcAatGcgAtcmCctTtgGa	74	56
ath-miR393b	gAtcAatGcgAtcmCctTtgGa	74	56
ath-miR394a	gGagGtgGacAgaAtgmCcaa	77	29
ath-miR394b	gGagGtgGacAgaAtgmCcaa	77	29
ath-miR395a	gAgtTccmCccAaamCacTtcAg	77	28
ath-miR395b	gagTccmCccmCaaAcacCttmCag	77	21
ath-miR395c	gagTccmCccmCaaAcacCttmCag	77	21
ath-miR395d	gAgtTccmCccAaamCacTtcAg	77	28
ath-miR395e	gAgtTccmCccAaamCacTtcAg	77	28
ath-miR395f	gagTccmCccmCaaAcacCttmCag	77	21
ath-miR396a	cagTtcAagAaaGctGtgGaa	70	35
ath-miR396b	aagTtcAagAaaGctGtgGaa	69	24
ath-miR397a	caTcaAcgmCtgmCacTcaAtga	73	39
ath-miR397b	caTcaAcgAtgmCacTcaAtga	70	35
ath-miR398a	aagGggTgamCctGagAacAca	80	39
ath-miR398b	cagGggTgamCctGagAacAca	81	51
ath-miR398c	cagGggTgamCctGagAacAca	81	51
ath-miR399a	cAggGcaAatmCtcmCttTggmCa	78	48
ath-miR399b	caGggmCaamCtcTccTttGgca	81	39
ath-miR399c	caGggmCaamCtcTccTttGgca	81	39
ath-miR399d	cggGgcAaaTctmCctTtgGca	79	47
ath-miR399e	cgaGgcAaaTctmCctTtgGca	76	41
ath-miR399f	cmCggGcaAatmCtcmCttTggmCa	80	41
ath-miR400	gTgamCttAtaAtamCtcTcaTa	63	32
ath-miR401	tgtmCggTcgAcacCcaGttTcg	78	59
ath-miR402	cAgaGgtTtaAtaGgcmCtcGaa	76	68
ath-miR403	cgAgtTtgTgcGtgAatmCtaa	71	46
ath-miR404	gmCtgmCcgmCaamCcgmCcaGcgTtaAt	88	55
ath-miR405a	agTtaTggGttAgamCccAacTcat	74	64
ath-miR405b	agTtaTggGttAgamCccAacTcat	74	64
ath-miR405d	agTtaTggGttAgamCccAacTcat	74	64
ath-miR406	ctGgaTtamCaaTagmCatTcta	67	38

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
ath-miR407	amCcaAaaGtaTatGatTtaAa	61	36
ath-miR408	gmCcaGggAagAggmCagTgcAt	87	35
ath-miR413	gtgmCagAacAagAgaAacTat	69	24
ath-miR414	tGacGatGatGatGaaGatGa	75	22
ath-miR415	atgTtcTgtTtcTgcTctGtt	68	15
ath-miR416	tGaamCagTgtAcgTacGaamCc	78	52
ath-miR417	tcgAacAaaTtcActAccTtc	65	21
ath-miR418	ggtmCagTtcAtcAtcAcaTta	66	22
ath-miR419	caamCatmCctmCagmCatTcaTaa	71	18
ath-miR420	tGcaTttmCcgTgaTtaGttTa	68	27
ath-miR426	cgtaaGgaaCaaAttTccAaaa	68	31
cbr-let-7	aamCtaTacAacmCtamCtamCctmCa	70	16
cbr-lin-4	tcamCacTtgAggTctmCagGga	78	70
cbr-l6	cggAatGcgTctmCatAcaAaa	71	40
cbr-miR-1	tamCatActTctTtamCatTcca	64	11
cbr-miR-124	tggmCatTcamCcgmCgtGccTta	80	43
cbr-miR-228	ccGtgAatTcaTgcAgtGccAtt	78	56
cbr-miR-230	ttTccTggTcgmCacAacTaaTac	74	27
cbr-miR-231	tccTgcmCtgTtgTtcAcgAgtTta	77	39
cbr-miR-232	tcAccGcaGttAagAtgmCatTta	71	44
cbr-miR-233	tccmCgcAcaTgcGcaTtgmCtcAa	83	59
cbr-miR-234	aAggGtaTtcTcgAgtAatAa	70	46
cbr-miR-236	agmCgtmCatTacmCtgAcaGtaTta	71	36
cbr-miR-239a	cmCagTacmCtaAttGtaGtamCaaa	68	44
cbr-miR-239b	caGtamCttTtgTgcAgtAcaa	68	51
cbr-miR-240	agcGaaAatTtgGagGccAgta	74	33
cbr-miR-241	tmCatTtcTcamCacmCtamCctmCa	74	7
cbr-miR-244	catAccActTtgTacAacmCaaAga	70	40
cbr-miR-245	gaGctActTggAggGgaaCcaAt	80	33
cbr-miR-246	aGctmCctAccmCaaTacAtgTaa	73	40
cbr-miR-248	tGagmCgtTatmCcgAgtAgtTgt	82	59
cbr-miR-249	gmCaamCacTcaAaaAtcmCtgTga	73	23
cbr-miR-250	cmCgtGccAacAgtTgamCtgTga	81	58
cbr-miR-251	aatAagAgtGgcAccActActTaa	74	41
cbr-miR-252	gttAccTgcGgcActActActTa	75	28

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
cbr-miR-253	agtTagTgtTagTgaGgtGtg	72	32
cbr-miR-254	tAtamCagTtgmCaaAagAttTgca	69	51
cbr-miR-259	aacmCagAttAggAtgAgaTtt	67	31
cbr-miR-268	amCcaAaamCtgmCttmCtaAttmCttGcc	73	23
cbr-miR-34	cAacmCagmCtaAccAcamCtgmCct	80	24
cbr-miR-35	cTtgmCaaGttTtcAccmCggTga	77	52
cbr-miR-353	gaTacmCaamCacAtgAtamCttg	68	23
cbr-miR-354	aggAgcAgcAacAaamCaaGgt	79	23
cbr-miR-355	catAgcTcaGgcTaaAacAaa	70	45
cbr-miR-356	ggAttTgtTcgmCgtTgcTcat	74	29
cbr-miR-357	tccGtcAatGacTggmCatTtt	73	52
cbr-miR-358	ccamCgamCtaAggAtamCcaAttg	72	26
cbr-miR-36	aTtgmCgaAttTtcAccmCggTga	76	44
cbr-miR-360	ttGtgAacGggAttAcgGtca	75	46
cbr-miR-38	aTacmCagGttGtcTccmCggTga	80	53
cbr-miR-39	cTaamCcgTttTtcAccmCggTga	76	49
cbr-miR-40	ctAgcTgaTtgAcamCccGgtGa	81	57
cbr-miR-41	tggGagTttTtcAccmCggTga	76	44
cbr-miR-42	cTgtAgaTgtTaamCccGgtg	76	39
cbr-miR-43	gcGacAgcAagTaaActGtgAta	74	32
cbr-miR-44	agcTgaAtgTgtmCtcTagTca	70	30
cbr-miR-45	agcTgaAtgTgtmCtcTagTca	70	30
cbr-miR-46	tgAagAgaGcgActmCcaTgamCa	79	33
cbr-miR-47	tGaaGagAgcGccTccAtgAca	80	38
cbr-miR-48	tcgmCatmCtamCtgAgcmCtamCctmCa	79	31
cbr-miR-49	tcTgcAgcTtcTcgTggTgcTt	80	36
cbr-miR-50	aamCccAagAatAtcAgamCatAtca	71	23
cbr-miR-51	aacAtgGcaAggAgcTacGggTa	80	34
cbr-miR-52	agmCacGgaAacAtaTgtAcgGgtg	81	44
cbr-miR-55	ctcGgcAgaAaaAtaTacGggTa	75	32
cbr-miR-57	acamCacAgcTcgAtcTacAggGta	78	47
cbr-miR-58	aTtgmCcgTacTgaAcgAtcTca	75	32
cbr-miR-60	tgGacTagAaaAtgTgcAtaAta	67	34
cbr-miR-61	gAgcAgaGtcAagGttmCtaGtca	74	53
cbr-miR-62	ctgTaaGctAgaTtamCatAtca	65	60

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
cbr-miR-64	tccGtamCacGctTcaGtgTcaTg	79	41
cbr-miR-67	tctActmCttTctAggAggTtgTga	73	54
cbr-miR-70	ctGggAacAccAatmCacGtaTta	74	29
cbr-miR-71	tcamCtamCccAtgTctTtca	67	20
cbr-miR-72	gmCtaTgcmCaamCatmCtgmCct	77	29
cbr-miR-73	amCtgAacTgcmCaamCatmCttGcca	79	44
cbr-miR-74	tctAgamCtgmCcaTttmCttGcca	74	28
cbr-miR-75	tGaaGgcGgtTggTagmCttTaa	79	48
cbr-miR-77	tggAcaGctAtgGccTgaTgaa	76	48
cbr-miR-79	aGctTtgGtaAccTagmCttTat	67	52
cbr-miR-80	tcGgcTttmCaamCtaAtgAtcTca	72	27
cbr-miR-81	acTagmCttTcamCgaTgaTctmCa	73	27
cbr-miR-82	amCtgGctTtcAcgAtgAtcTca	73	30
cbr-miR-83	acamCtgAatTtaTatGgtGcta	67	47
cbr-miR-84	gacAgcAttGcaAacTacmCtca	73	36
cbr-miR-85	gmCacGccTttTcaAatActTtgTa	71	33
cbr-miR-86	gActGtgGcaAagmCatTcamCtta	73	44
cbr-miR-87	amCacmCtgAaamCttTgcTcac	72	20
cbr-miR-90	gGggmCatTcaAacAacAtaTca	73	23
cel-let-7	aamCtaTacAacmCtamCtamCctmCa	70	16
cel-lin-4	tcamCacTtgAggTctmCagGga	78	70
cel-l6	cgaAatGcgTctmCatAcaAaa	69	44
cel-miR-1	tamCatActTctTtamCatTcca	64	11
cel-miR-124	tggmCatTcamCcgmCgtGccTta	80	43
cel-miR-2	gcAcaTcaAagmCtgGctGtgAta	75	68
cel-miR-227	gttmCagAatmCatGtcGaaAgct	71	34
cel-miR-228	ccGtgAatTcaTgcAgtGccAtt	78	56
cel-miR-229	acgAtgGaaAagAtaAccAgtGtcAtt	74	43
cel-miR-230	tcTccTggTcgmCacAacTaaTac	76	27
cel-miR-231	ttcTgcmCtgTtgAtcAcgAgtTta	75	46
cel-miR-232	tcAccGcaGttAagAtgmCatTta	71	44
cel-miR-233	tccmCgcAcaTgcGcaTtgmCtcAa	83	59
cel-miR-234	aAggGtaTtcTcgAgtAatAa	70	46
cel-miR-235	tcAggmCcgGggAgaGtgmCaaTa	85	39
cel-miR-236	agmCgtmCatTacmCtgAcaGtaTta	71	36

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
cel-miR-237	aAgcTgtTcgAgaAttmCtcAggGa	78	54
cel-miR-238	tcTgaAtgGcaTcgGagTacAaa	75	34
cel-miR-239a	ccaGtamCctAtgTgtAgtAcaAa	71	50
cel-miR-239b	cAgtActTttGtgTagTacAa	68	45
cel-miR-240	agcGaaGatTtgGggGccAgta	80	33
cel-miR-241	tmCatTtcTcgmCacmCtamCctmCa	76	18
cel-miR-242	tmCgaAgcAaaGgcmCtamCgcAa	82	49
cel-miR-243	gatAtcmCcgmCcgmCgaTcgTacmCg	84	58
cel-miR-244	catAccActTtgTacAacmCaaAga	70	40
cel-miR-245	gaGctActTggAggGgatCcaAt	80	33
cel-miR-246	aGctmCctAccmCgaAacAtgTaa	75	30
cel-miR-247	aAgaAgaGaaTagGctmCtaGtca	71	50
cel-miR-248	tGagmCgtTatmCcgTgcAcgTgt	82	48
cel-miR-249	gcaAcmCtcAaaAgtmCctGtga	74	35
cel-miR-250	cmCatGccAacAgtTgamCtgTga	79	58
cel-miR-251	aatAagAgaGgcAccActActTaa	74	41
cel-miR-252	gttAccTgcGgcActActActTa	75	28
cel-miR-253	ggTcaGtgTtaGtgAggTgt	74	20
cel-miR-254	cmCtamCagTcgmCgaAagAttTgca	76	44
cel-miR-256	tacAgtmCttmCtaTgcAttmCca	69	32
cel-miR-257	tcActGggTacTccTgaTacTc	76	42
cel-miR-258	aaaAggAttmCctmCtcAaaAcc	67	45
cel-miR-259	tacmCagAttAggAtgAgaTtt	67	30
cel-miR-260	ctamCaaGagTtcGacAtcAc	70	34
cel-miR-261	cgtGaaAacTaaAaaGcta	61	24
cel-miR-262	aTcaGaaAacAtcGagAaac	67	25
cel-miR-264	catAacAacAacmCacmCcgmCc	77	18
cel-miR-265	atamCcamCccTtcmCtcmCctmCa	77	6
cel-miR-266	gctTtgmCcaAagTctTgcmCt	74	44
cel-miR-267	tgcAgtAgamCacTtcAcgGg	81	29
cel-miR-268	amCcaAacTgcTtcTaaTtcTtgmCc	74	19
cel-miR-269	aGttTtgmCcaGagTctTgccc	74	49
cel-miR-270	cTccActGctAcaTcaTgccc	75	27
cel-miR-271	aaTgcTttmCccAccmCggmCga	82	33
cel-miR-272	cAaamCacmCcaTgcmCtamCa	75	20

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
cel-miR-273	cAgcmCgamCacAgtAcgGgca	85	37
cel-miR-34	cAacmCagmCtaAccAcamCtgmCct	80	24
cel-miR-35	amCtgmCtaGttTccAccmCggTga	80	39
cel-miR-353	aaTacmCaamCacAtgGcaAttg	70	33
cel-miR-354	aggAgcAgcAacAaamCaaGgt	79	23
cel-miR-355	catAgcTcaGgcTaaAacAaa	70	45
cel-miR-356	tgAttTgtTcgmCgtTgcTcaa	73	29
cel-miR-357	tmCctGcaAcgActGgcAttTa	77	33
cel-miR-358	ccTtgAcaGggAtamCcaAttg	72	42
cel-miR-359	tmCgtmCagAgaAagAccAgtGa	78	25
cel-miR-36	cAtgmCgaAttTtcAccmCggTga	77	44
cel-miR-360	ttGtgAacGggAttAcgGtca	75	46
cel-miR-37	amCtgmCaaGtgTtcAccmCggTga	82	46
cel-miR-38	amCtcmCagTttTtcTccmCggTga	77	28
cel-miR-39	cAagmCtgAttTacAccmCggTga	77	38
cel-miR-392	tcAtcAcamCgtGatmCgaTgaTa	75	59
cel-miR-40	tTagmCtgAtgTacAccmCggTga	78	52
cel-miR-41	tAggTgaTttTtcAccmCggTga	76	44
cel-miR-42	cTgtAgaTgtTaamCccGgtg	76	39
cel-miR-43	gcGacAgcAagTaaActGtgAta	74	32
cel-miR-44	agcTgaAtgTgtmCtcTagTca	70	30
cel-miR-45	agcTgaAtgTgtmCtcTagTca	70	30
cel-miR-46	tgAagAgaGcgActmCcaTgamCa	79	33
cel-miR-47	tGaaGagAgcGccTccAtgAca	80	38
cel-miR-48	tcgmCatmCtamCtgAgcmCtamCctmCa	79	31
cel-miR-49	tcTgcAgcTtcTcgTggTgcTt	80	36
cel-miR-50	aamCccAagAatAccAgamCatAtca	73	16
cel-miR-51	aacAtgGatAggAgcTacGggTa	79	31
cel-miR-52	agmCacGgaAacAtaTgtAcgGgtg	81	44
cel-miR-53	agmCacGgaAacAaaTgtAcgGgtg	82	33
cel-miR-54	cTcgGatTatGaaGatTacGggTa	75	35
cel-miR-55	ctcAgcAgaAacTtaTacGggTa	74	33
cel-miR-56	ctcAgcGgaAacAttAcgGgtg	77	25
cel-miR-56*	tacAacmCcaAaaTggAtcmCgcmCa	78	42
cel-miR-57	acacmCacAgtTcgAtcTacAggGta	78	47

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
cel-miR-58	aTtgmCcgTacTgaAcgAtcTca	75	32
cel-miR-59	cAtcAtcmCtgAtaAacGatTcga	70	35
cel-miR-60	tgAacTagAaaAtgTgcAtaAta	65	34
cel-miR-61	gagAtgAgtAacGgtTctAgtmCa	75	52
cel-miR-62	ctgTaaGctAgaTtamCatAtca	65	60
cel-miR-63	ttTccAacTcgmCttmCagTgtmCata	75	31
cel-miR-64	ttcGgtAacGctTcaGtgTcaTa	76	41
cel-miR-65	ttcGgtTacGctTcaGtgTcaTa	75	41
cel-miR-66	tmCacAtcmCctAatmCagTgtmCatg	75	27
cel-miR-67	tctActmCttTctAggAggTtgTga	73	54
cel-miR-68	tmCtamCacTttTgaGtcTtcGa	69	33
cel-miR-69	tcTacActTttTaaTttTcga	59	20
cel-miR-70	atgGaaAcamCcaAcgAcgTatTa	73	33
cel-miR-71	tcamCtamCccAtgTctTtca	67	20
cel-miR-72	gmCtaTgcmCaamCatmCttGcct	76	34
cel-miR-73	actGaamCtgmCctAcaTctTgcmCa	79	28
cel-miR-74	tgTagActGccAttTctTgcmCa	76	43
cel-miR-75	tgAagmCcgGttGgtAgcTttAa	77	48
cel-miR-76	tcaAggmCttmCatmCaamCaamCgaa	75	31
cel-miR-77	tggAcaGctAtgGccTgaTgaa	76	48
cel-miR-78	gcamCaaAcaAccAggmCctmCca	79	38
cel-miR-79	aGctTtgGtaAccTagmCttTat	67	52
cel-miR-80	tcGgcTttmCaamCtaAtgAtcTca	72	27
cel-miR-81	acTagmCttTcamCgaTgaTctmCa	73	27
cel-miR-82	amCtgGctTtcAcgAtgAtcTca	73	30
cel-miR-83	ttamCtgAatTtaTatGgtGcta	65	33
cel-miR-84	tamCaaTatTacAtamCtamCctmCa	66	26
cel-miR-85	gmCacGacTttTcaAatActTtgTa	70	35
cel-miR-86	gActGtgGcaAagmCatTcamCtta	73	44
cel-miR-87	amCacmCtgAaamCttTgcTcac	72	20
cel-miR-90	gGggmCatTcaAacAacAtaTca	73	23
dme-bantam	aaTcaGctTtcAaaAtgAtcTca	66	40
dme-let-7	amCtaTacAacmCtamCtamCctmCa	71	16
dme-miR-1	ctcmCatActTctTtamCatTcca	67	11
dme-miR-10	acaAatTcgGatmCtamCagGgt	73	37

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
dme-miR-100	cAcaAgtTcgGatTtamCggGtt	74	48
dme-miR-11	gcaAgaActmCagActGtgAtg	71	40
dme-miR-12	accAgtAccTgaTgtAatActmCa	73	33
dme-miR-124	ctTggmCatTcamCggmCgtGccTta	81	43
dme-miR-125	tcamCaaGttAggGtcTcaGgga	77	35
dme-miR-133	acAgcTggTtgAagGggAccAa	82	41
dme-miR-13a	acTcaTcaAaaTggmCtgTgaTa	72	34
dme-miR-13b	acTcgTcaAaaTggmCtgTgaTa	74	34
dme-miR-14	tAggAgaGagAaaAagActGa	71	15
dme-miR-184	gcmCctTatmCagTtcTccGtcmCa	77	23
dme-miR-184*	cGggGcgAgaGaaTgaTaaGg	83	19
dme-miR-210	tAgcmCgcTgtmCacAcgmCacAa	84	37
dme-miR-219	cAagAatTgcGttTggAcaAtca	72	35
dme-miR-263a	gtgAatTctTccAgtGccAttAac	72	37
dme-miR-263b	gTgaAttmCtcmCcaGtgmCcaAg	77	34
dme-miR-274	aTtamCccGttAgtGtcGgtmCacAaaa	79	51
dme-miR-275	cGcgmCgcTacTtcAggTacmCtga	82	64
dme-miR-276a	agAgcAcgGtaTgaAgtTccTa	75	33
dme-miR-276a*	cgtAggAacTctAtamCctmCgcTg	76	30
dme-miR-276b	agAgcAcgGtaTtaAgtTccTa	71	40
dme-miR-276b*	cgtAggAacTctAtamCctmCgcTg	76	30
dme-miR-277	tgTcgTacmCagAtaGtgmCatTta	72	38
dme-miR-278	aaAcgGacGaaAgtmCccAccGa	80	41
dme-miR-279	tTaaTgaGtgTggAtcTagTca	70	40
dme-miR-280	tAtcAttTcaTatGcaAcgTaaAtamCa	70	40
dme-miR-281	acAaaGagAgcAatTccAtgAca	74	26
dme-miR-281-1*	actGtcGacGgacGagmCtcTctt	80	56
dme-miR-281-2*	actGtcGacGgaTagmCtcTctt	77	56
dme-miR-282	amCagAcaAagmCctAgtAgaGgcTagAtt	80	49
dme-miR-283	aGaaTtamCcaGctGatAttTa	67	54
dme-miR-284	caAttGctGgaAtcAagTtgmCtgActTca	78	45
dme-miR-285	gcamCtgAttTcgAatGgtGcta	74	55
dme-miR-286	agcAcgAgtGttmCggTctAgtmCa	80	46
dme-miR-287	gtgmCaaAcgAttTtcAacAca	68	27
dme-miR-288	caTgaAatGaaAtcGacAtgAaa	68	27

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
dme-miR-289	agtmCgcAggmCtcmCacTtaAatAttTa	74	42
dme-miR-2a	gcTcaTcaAagmCtgGctGtgAta	75	68
dme-miR-2b	gcTccTcaAagmCtgGctGtgAta	76	62
dme-miR-2c	gcmCcaTcaAagmCtgGctGtgAta	78	68
dme-miR-3	tgaGacAcamCttTgcmCcaGtga	77	45
dme-miR-303	accAgtTtcmCtgTgaAacmCtaAa	72	45
dme-miR-304	ctcAcaTttAcaAatTgaGatTa	64	55
dme-miR-305	cagAgcAccTgaTgaAgtAcaAt	74	31
dme-miR-306	tTgaGagTcamCtaAgtAccTga	72	42
dme-miR-306*	gmCacAggmCacAgaGtgAccmCcc	86	37
dme-miR-307	ctmCacTcaAggAggTtgTga	74	33
dme-miR-308	cTcamCagTatAatmCctGtgAtt	69	64
dme-miR-309	tAggAcaAacTttAccmCagTgc	74	37
dme-miR-310	aAagGccGggAagTgtGcaAta	79	28
dme-miR-311	tmCagGccGgtGaaTgtGcaAta	81	36
dme-miR-312	tmCagGccGtcTcaAgtGcaAta	77	39
dme-miR-313	tcgGgcTgtGaaAagTgcAata	77	29
dme-miR-314	cmCgaActTatTggmCtcGaaTa	72	30
dme-miR-315	gmCttTctGagmCaamCaaTcaAaa	72	37
dme-miR-316	cgcgcagTaaGcgGaaAaaGaca	76	35
dme-miR-317	amCtgGatAccAccAgtTgtGttmCa	82	47
dme-miR-318	tgaGatAaamCaaAgcmCcaGtga	73	25
dme-miR-31a	tcaGctAtgmCcgAcaTctTgcmCa	80	45
dme-miR-31b	cagmCtaTtcmCgacCatmCttGcca	75	31
dme-miR-33	cAatGcgActAcaAtgmCacmCt	75	26
dme-miR-34	cAacmCagmCtaAccAcamCtgmCca	80	24
dme-miR-4	tcAatGgtTgtmCtaGctTtat	67	34
dme-miR-5	catAtcAcaAcgAtcGttmCctTt	69	54
dme-miR-6	aaaAagAacAgcmCacTgtGata	71	36
dme-miR-7	amCaamCaaAatmCacTagTctTcca	71	30
dme-miR-79	atgmCttTggTaaTctAgtTta	66	34
dme-miR-8	gamCatmCttTacmCtgAcaGtaTta	67	36
dme-miR-87	camCacmCtgAaaTttTgcTcaa	69	32
dme-miR-92a	aTagGccGggAcaAgtGcaAtg	80	28
dme-miR-92b	gmCagGccGggActAgtGcaAtt	83	36

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
dme-miR-9a	tcAtamCagmCtaGatAacmCaaAga	71	34
dme-miR-9b	catAcaGctAaaAtcAccAaaGa	69	24
dme-miR-9c	tctAcaGctAgaAtamCcaAaga	68	27
dme-miR-iab-4-3p	gttAcgTatActGaaGgtAtamCcg	73	59
dme-miR-iab-4-5p	tmCagGatAcaTtcAgtAtamCgt	72	34
dps-bantam	aaTcaGctTtcAaaAtgAtcTca	66	40
dps-let-7	amCtaTacAacmCtamCtamCctmCa	71	16
dps-miR-1	ctcmCatActTctTtamCatTcca	67	11
dps-miR-10	acaAatTcgGatmCtamCagGgt	73	37
dps-miR-100	cacAagTtcGgaAttAcgGgtt	74	50
dps-miR-11	gcaAgaActmCagActGtgAtg	71	40
dps-miR-12	accAgtAccTgaTgtAatActmCa	73	33
dps-miR-124	ctTggmCatTcamCcgmCgtGccTta	81	43
dps-miR-125	tcamCaaGttAggGtcTcaGgga	77	35
dps-miR-133	acAgcTggTtgAagGggAccAa	82	41
dps-miR-13a	acTcaTcaAaaTggmCtgTgaTa	72	34
dps-miR-13b	acTcgTcaAaaTggmCtgTgaTa	74	34
dps-miR-14	tAggAgaGagAaaAagActGa	71	15
dps-miR-184	gcmCctTatmCagTtcTccGtcmCa	77	23
dps-miR-210	tAgcmCgcTgtmCacAcgmCacAa	84	37
dps-miR-219	cAagAatTgcGttTggAcaAtca	72	35
dps-miR-263a	gtgAatTctTccAgtGccAttAac	72	37
dps-miR-263b	gTgaAttmCtcmCcaGtgmCcaAg	77	34
dps-miR-274	aTtamCccGttAgtGtcGgtmCacAaaa	79	51
dps-miR-275	cGcgmCgcTacTtcAggTacmCtga	82	64
dps-miR-276a	agAgcAcgGtaTgaAgtTccTa	75	33
dps-miR-276b	agAgcAcgGtaTtaAgtTccTa	71	40
dps-miR-277	tgTcgTacmCagAtaGtgmCatTta	72	38
dps-miR-278	aAacGgamCgaAagTccmCtcmCga	81	53
dps-miR-279	tTaaTgaGtgTggAtcTagTca	70	40
dps-miR-280	tAtcAttTcaTatGcaAcgTaaAtamCa	70	40
dps-miR-281	acAaaGagAgcAatTccAtgAca	74	26
dps-miR-282	amCagAcaAagmCctAgtAgaGgcTagAtt	80	49
dps-miR-283	aGaaTtamCcaGctGatAttTa	67	54
dps-miR-284	caAttGctGgaAtcAagTtgmCtgActTca	78	45

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
dps-miR-285	gcamCtgAttTcgAatGgtGcta	74	55
dps-miR-286	agcAcgAgtGttmCggTctAgtmCa	80	46
dps-miR-287	gtgmCaaAcgAttTtcAacAca	68	27
dps-miR-288	caTgaAatGaaAtcGacAtgAaa	68	27
dps-miR-289	agtmCgcAggmCtcmCacTtaAatAttTa	74	42
dps-miR-2a	gcTcaTcaAagmCtgGctGtgAta	75	68
dps-miR-2b	gcTccTcaAagmCtgGctGtgAta	76	62
dps-miR-2c	gcmCcaTcaAagmCtgGctGtgAta	78	68
dps-miR-3	tgaGacAcamCttTgcmCcaGtga	77	45
dps-miR-304	ctcAcaTttAcaAatTgaGatTa	64	55
dps-miR-305	cagAgcAccTgaTgaAgtAcaAt	74	31
dps-miR-306	tTgaGagTcamCtaAgtAccTga	72	42
dps-miR-307	ctmCacTcaAggAggTtgTga	74	33
dps-miR-308	cTcamCagTatAatmCctGtgAtt	69	64
dps-miR-309	tAagAcaAacTtcAccmCagTgc	74	29
dps-miR-314	cmCgaActTatTggmCtcGaaTa	72	30
dps-miR-315	gmCttTctGagmCaamCaaTcaAaa	72	37
dps-miR-316	cgcgcagTaaGcgGaaAaaGaca	76	35
dps-miR-317	aTtgGatAccAccAgtTgtGttmCa	79	47
dps-miR-318	tgaGatAaamCaaAgcmCcaGtga	73	25
dps-miR-31a	tcaGctAtgmCcgAcaTctTgcmCa	80	45
dps-miR-31b	tcaGctAttmCcgAcaTctTgcmCa	77	31
dps-miR-33	cAatGcgActAcaAtgmCacmCt	75	26
dps-miR-34	cAacmCagmCtaAccAcamCtgmCca	80	24
dps-miR-4	tcAatGgtTgtmCtaGctTtat	67	34
dps-miR-5	catAtcAcaAcgAtcGttmCctTt	69	54
dps-miR-6	aaaAagAacAgcmCacTgtGata	71	36
dps-miR-7	amCaamCaaAatmCacTagTctTcca	71	30
dps-miR-79	atgmCttTggTaaTctAgtTtta	66	34
dps-miR-8	gamCatmCttTacmCtgAcaGtaTta	67	36
dps-miR-87	camCacmCtgAaaTttTgcTcaa	69	32
dps-miR-92a	aTagGccGggAcaAgtGcaAtg	80	28
dps-miR-92b	gmCagGccGggActAgtGcaAtt	83	36
dps-miR-9a	tcAtamCagmCtaGatAacmCaaAga	71	34
dps-miR-9b	catAcaGctAaaAtcAccAaaGa	69	24

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
dps-miR-9c	tctAcaGctAgaAtamCcaAaga	68	27
dps-miR-iab-4-3p	gttAcgTatActGaaGgtAtamCcg	73	59
dps-miR-iab-4-5p	tmCagGatAcaTtcAgtAtamCgt	72	34
dre-miR-10a	cAcaAatTcgGatmCtamCagGgtA	74	37
dre-miR-10b	amCaaAttmCggTtcTacAggGta	73	35
dre-miR-181b	cccAccGacAgcAatGaaTgtt	78	30
dre-miR-182	tgtGagTtcTacmCatTgcmCaaa	72	32
dre-miR-182*	taGttGgcAagTctAgaAcca	72	32
dre-miR-183	caGtgAatTctAccAgtGccAta	73	32
dre-miR-187	ggcTgcAacAcaAgamCacGa	79	30
dre-miR-192	ggcTgtmCaaTtcAtaGgtmCat	72	46
dre-miR-196a	ccaAcaAcaTgaAacTacmCta	67	20
dre-miR-199a	gaAcaGgtAgtmCtgAacActGgg	78	40
dre-miR-203	cAagTggTccTaaAcaTttmCac	70	31
dre-miR-204	aggmCatAggAtgAcaAagGgaa	75	25
dre-miR-205	caGacTccGgtGgaAtgAagGa	81	39
dre-miR-210	ttAgcmCgcTgtmCacAcgmCacAg	85	37
dre-miR-213	gGtamCaaTcaAcgGtcAatGgt	75	43
dre-miR-214	ctGccTgtmCtgTgcmCtgmCtgt	81	30
dre-miR-216	camCagTtgmCcaGctGagAtta	74	64
dre-miR-217	atcmCaaTcaGttmCctGatGcaGta	75	49
dre-miR-219	agAatTgcGttTggAcaAtca	70	35
dre-miR-220	aAgtGtcmCgaTacGgtTgtGg	81	47
dre-miR-221	gAaamCccAgcAgamCaaTgtAgct	80	31
dre-miR-222	gaGacmCcaGtaGccAgaTgtAgct	80	38
dre-miR-223	gGggTatTtgAcaAacTgamCa	73	40
dre-miR-34a	aamCaamCcaGctAagAcamCtgmCca	80	27
dre-miR-7	caamCaaAatmCacTagTctTcca	69	30
dre-miR-7b	aamCaaAatmCacAagTctTcca	68	24
ebv-miR-B	aGcamCgtmCacTtcmCacTaaGa	77	25
ebv-miR-B	gcAagGgcGaaTgcAgaAaaTa	78	27
ebv-miR-BHRF1-1	aacTccGggGctGatmCagGtta	80	50
ebv-miR-BHRF1-2	tTcaAttTctGccGcaAaaGata	70	52
ebv-miR-BHRF1-2*	gctAtcTgcTgcAacAgaAttt	71	62
ebv-miR-BHRF1-3	gtGtgmCttAcamCacTtcmCcgTta	76	47

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
gga-let-7a	aamCtaTAcAacmCtamCtamCctmCa	70	16
gga-let-7b	aamCcamCacAacmCtamCtamCctmCa	77	6
gga-let-7c	aamCcaTAcAacmCtamCtamCctmCa	74	11
gga-let-7d	actAtgmCaamCccActAccTct	74	24
gga-let-7f	aamCtaTAcAatmCtamCtamCctmCa	67	16
gga-let-7g	amCtgTAcAaamCtamCtamCctmCa	71	30
gga-let-7i	amCagmCacAaamCtamCtamCctmCa	76	18
gga-let-7j	aamCtaTAcAacmCtamCtamCctmCa	70	16
gga-let-7k	aActAttmCaaTctActAccTca	67	22
gga-miR-1	tamCatActTctTtamCatTcca	64	11
gga-miR-100	cacAagTtcGgaTctAcgGgtt	77	38
gga-miR-101	cttmCagTtaTcamCagTacTgta	68	54
gga-miR-103	tmCatAgcmCctGtamCaaTgcTgct	80	63
gga-miR-106	tacmCtgmCacTgtAagmCacTttt	72	37
gga-miR-107	tGatAgcmCctGtamCaaTgcTgct	80	63
gga-miR-10b	amCaaAttmCggTtcTacAggGta	73	35
gga-miR-122a	acAaamCacmCatTgtmCacActmCca	78	25
gga-miR-124a	tggmCatTcamCcgmCgtGccTtaa	80	43
gga-miR-124b	tggmCatTcamCtgmCgtGccTtaa	77	48
gga-miR-125b	tcamCaaGttAggGtcTcaGgga	77	35
gga-miR-126	gcAttAttActmCacGgtAcga	71	25
gga-miR-128a	aaAagAgamCcgGttmCacTgtGa	77	47
gga-miR-128b	aaAagAgamCcgGttmCacTgtGa	77	47
gga-miR-130a	atgmCccTttTaaTatTgcActg	68	42
gga-miR-130b	acGccmCttTcaTtaTtgmCacTg	75	26
gga-miR-133a	acAgcTggTtgAagGggAccAa	82	41
gga-miR-133b	taGctGgtTgaAggGgaaCcaa	81	37
gga-miR-133c	gcAgcTggTtgAagGggAccAa	83	41
gga-miR-135a	tcamCatAggAatAaaAagmCcaTa	69	22
gga-miR-137	cTacGcgTatTctTaaGcaAta	68	48
gga-miR-138	gatTcamCaamCacmCagmCt	70	24
gga-miR-140	ctAccAtaGggTaaAacmCact	71	43
gga-miR-142-3p	tmCcaTaaAgtAggAaamCacTaca	72	29
gga-miR-142-5p	gtaGtgmCttTctActTtaTg	63	36
gga-miR-146	aAccmCatGgaAttmCagTtcTca	73	44

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
gga-miR-148a	acaAagTtcTgtAgtGcamCtga	72	54
gga-miR-153	tcamCttTtgTgamCtaTgcAa	68	35
gga-miR-155	ccmCctAtcAcgAttAgtAttAa	71	29
gga-miR-15a	cAcaAacmCatTatGtgmCtgmCta	73	35
gga-miR-15b	tgcAaamCcaTgaTgtGctGcta	77	52
gga-miR-16	cacmCaaTatTtamCgtGctGcta	71	38
gga-miR-17-3p	acAagTgcmCttmCacTgcAgt	77	47
gga-miR-17-5p	actAccTgcActGtaAgcActTtg	74	39
gga-miR-181a	acTcamCcgAcaGcgTtgAatGtt	77	49
gga-miR-181b	cccAccGacAgcAatGaaTgtt	78	30
gga-miR-183	caGtgAatTctAccAgtGccAta	73	32
gga-miR-184	acmCctTatmCagTtcTccGtcmCa	76	23
gga-miR-187	ggcTgcAacAcaAgamCacGa	79	30
gga-miR-18a	tatmCtgmCacTagAtgmCacmCtta	71	40
gga-miR-18b	taamCtgmCacTagAtgmCacmCtta	72	40
gga-miR-190	acmCtaAtaTatmCaaAcaTatmCa	62	31
gga-miR-194	tccAcaTggAgtTgcTgtTaca	75	41
gga-miR-196	ccaAcaAcaTgaAacTacmCta	67	20
gga-miR-199a	gaAcaGgtAgtmCtgAacActGgg	78	40
gga-miR-19a	tmCagTttTgcAtaGatTtgmCaca	72	37
gga-miR-19b	tmCagTttTgcAtgGatTtgmCaca	75	34
gga-miR-1b	tacAtamCttmCttAacAttmCca	64	16
gga-miR-20	ctAccTgcActAtaAgtActTta	70	26
gga-miR-200a	acaTcgTtamCcaGacAgtGtta	72	39
gga-miR-200b	atcAtcAttAccAggmCagTatTa	70	29
gga-miR-203	cAagTggTccTaaAcaTttmCac	70	31
gga-miR-204	aggmCatAggAtgAcaAagGgaa	75	25
gga-miR-205a	caGacTccGgtGgaAtgAagGa	81	39
gga-miR-205b	cAgaTtcmCggTggAatGaaGgg	80	55
gga-miR-206	ccamCacActTccTtamCatTcca	73	11
gga-miR-213	gGtamCaaTcaAgtGtcGatGgt	79	67
gga-miR-215	gtcTgtmCaaTtcAtaGgtmCat	70	50
gga-miR-216	camCagTtgmCcaGctGagAtta	74	64
gga-miR-217	atcmCaaTcaGttmCctGatGcaGta	75	49
gga-miR-218	acAtgGttAgaTcaAgcAcaa	70	40

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
gga-miR-219	agAatTgcGttTggAcaAtca	70	35
gga-miR-221	gAaamCccAgcAgamCaaTgtAgct	80	31
gga-miR-222a	gaGacmCcaGtaGccAgaTgtAgct	80	38
gga-miR-222b	gAgamCccAgtAgcmCagAtgTagTt	80	28
gga-miR-223	gGggTatTtgAcaAacTgamCa	73	40
gga-miR-23b	ggtAatmCccTggmCaaTgtGat	76	38
gga-miR-24	cTgtTccTgcTgaActGagmCca	80	35
gga-miR-26a	gcmCtaTccTggAttActTgaa	70	34
gga-miR-27b	gcAgaActTagmCcamCtgTgaa	74	38
gga-miR-29a	aamCcgAttTcaAatGgtGcta	71	47
gga-miR-29b	aamCacTgaTttmCaaAtgGtgmCta	71	47
gga-miR-29c	amCcgAttTcaAatGgtGcta	71	47
gga-miR-301	atGctTtgAcaAtaTtaTtgmCacTg	70	45
gga-miR-302	tcActAaaAcaTggAagmCacTt	71	23
gga-miR-30a-3p	gctGcaAacAtcmCgammCtgAaag	74	28
gga-miR-30a-5p	cTtcmCagTcgAggAtgTttAca	73	31
gga-miR-30b	agcTgaGtgTagGatGttTaca	71	33
gga-miR-30c	gmCtgAgaGtgTagGatGttTaca	73	33
gga-miR-30d	cttmCcaGtcGggGatGttTaca	76	44
gga-miR-30e	tcmCagTcaAggAtgTttAca	69	30
gga-miR-31	cagmCtaTgcmCaamCatmCttGcct	77	34
gga-miR-32	gcaActTagTaaTgtGcaAta	65	43
gga-miR-33	cAatGcaActAcaAtgmCac	68	30
gga-miR-34a	aamCaamCcaGctAagAcamCtgmCca	80	27
gga-miR-34b	caAtcAgcTaamCtamCacTgcmCtg	75	32
gga-miR-34c	gcAatmCagmCtaActAcamCtgmCct	76	31
gga-miR-7	caamCaaAatmCacTagTctTcca	69	30
gga-miR-7b	aacAaaAatmCacTagTctTcca	66	30
gga-miR-9	tcAtamCagmCtaGatAcacmCaaAga	71	34
gga-miR-92	cagGccGggAcaAgtGcaAta	79	28
gga-miR-99a	cacAagAtcGgaTctAcgGtt	77	42
hsa-let-7a	aamCtaTacAcacmCtamCtamCctmCa	70	16
hsa-let-7b	aamCcamCacAcacmCtamCtamCctmCa	77	6
hsa-let-7c	aamCcaTacAcacmCtamCtamCctmCa	74	11
hsa-let-7d	actAtgmCaamCctActAccTct	71	24

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
hsa-let-7e	actAtamCaamCctmCctAccTca	71	16
hsa-let-7f	aamCtaTacAatmCtamCtamCctmCa	67	16
hsa-let-7g	amCtgTacAaamCtamCtamCctmCa	71	30
hsa-let-7i	amCagmCacAaamCtamCtamCctmCa	76	18
hsa-miR-1	tamCatActTctTtamCatTcca	64	11
hsa-miR-100	cacAagTtcGgaTctAcgGgtt	77	38
hsa-miR-101	cttmCagTtaTcamCagTacTgta	68	54
hsa-miR-103	tmCatAgcmCctGtamCaaTgcTgct	80	63
hsa-miR-105	acAggAgtmCtgAgcAttTga	73	33
hsa-miR-106a	gctAccTgcActGtaAgcActTtt	75	37
hsa-miR-106b	atcTgcActGtcAgcActTta	72	35
hsa-miR-107	tGatAgcmCctGtamCaaTgcTgct	80	63
hsa-miR-108	aatGccmCctAaaAatmCctTat	66	23
hsa-miR-10a	cAcaAatTcgGatmCtamCagGgtt	74	37
hsa-miR-10b	amCaaAttmCggTtcTacAggGta	73	35
hsa-miR-122a	acAaamCacmCatTgtmCacActmCca	78	25
hsa-miR-124a	tggmCatTcamCcgmCgtGccTtaa	80	43
hsa-miR-125a	cAcaGgtTaaAggGtcTcaGgga	79	35
hsa-miR-125b	tcamCaaGttAggGtcTcaGgga	77	35
hsa-miR-126	gcAttAttActmCacGgtAcga	71	25
hsa-miR-126*	cgmCgtAccAaaAgtAatAatg	68	28
hsa-miR-127	agcmCaaGctmCagAcgGatmCcga	81	54
hsa-miR-128a	aaAagAgamCcgGttmCacTgtGa	77	47
hsa-miR-128b	gaAagAgamCcgGttmCacTgtGa	78	47
hsa-miR-129	gcAagmCccAgamCcgmCaaAaag	80	21
hsa-miR-130a	aTgcmCctTttAacAttGcamCtg	74	42
hsa-miR-130b	aTgcmCctTtcAtcAttGcamCtg	75	34
hsa-miR-132	cgAccAtgGctGtaGacTgtTa	76	48
hsa-miR-133a	acAgcTggTtgAagGggAccAa	82	41
hsa-miR-133b	taGctGgtTgaAggGgaaCcaa	81	37
hsa-miR-134	ccmCtcTggTcaAccAgtmCaca	77	57
hsa-miR-135a	tcamCatAggAatAaaAagmCcaTa	69	22
hsa-miR-135b	cacAtaGgaAtgAaaAgcmCata	70	22
hsa-miR-136	tccAtcAtcAaaAcaAatGgaGt	71	25
hsa-miR-137	cTacGcgTatTctTaaGcaAta	68	48

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
hsa-miR-138	gatTcamCaamCacmCagmCt	70	24
hsa-miR-139	aGacAcgTgcActGtaGa	75	42
hsa-miR-140	ctAccAtaGggTaaAacmCact	71	43
hsa-miR-141	cmCatmCttTacmCagAcaGtgTta	70	33
hsa-miR-142-3p	tmCcaTaaAgtAggAaamCacTaca	72	29
hsa-miR-142-5p	gtaGtgmCttTctActTtaTg	63	36
hsa-miR-143	tGagmCtamCagTgcTtcAtcTca	75	56
hsa-miR-144	ctaGtamCatmCatmCtaTacTgta	64	37
hsa-miR-145	aAggGatTccTggGaaAacTggAc	79	50
hsa-miR-146	aAccmCatGgaAttmCagTtcTca	73	44
hsa-miR-147	gcAgaAgcAttTccAcamCac	74	25
hsa-miR-148a	acaAagTtcTgtAgtGcamCtga	72	54
hsa-miR-148b	acaAagTtcTgtGatGcamCtga	72	39
hsa-miR-149	ggaGtgAagAcamCggAgcmCaga	80	31
hsa-miR-150	cacTggTacAagGgtTggGaga	78	30
hsa-miR-151	ccTcaAggAgcTtcAgtmCtaGt	75	45
hsa-miR-152	cmCcaAgtTctGtcAtgmCacTga	78	36
hsa-miR-153	tcamCttTtgTgamCtaTgcAa	68	35
hsa-miR-154	cGaaGgcAacAcgGatAacmCta	78	40
hsa-miR-154*	aAtaGgtmCaamCcgTgtAtgAtt	74	40
hsa-miR-155	ccmCctAtcAcgAttAgcAttAa	71	29
hsa-miR-15a	cAcaAacmCatTatGtgmCtgmCta	73	35
hsa-miR-15b	tgtAaamCcaTgaTgtGctGcta	74	38
hsa-miR-16	cgmCcaAtaTttAcgTgcTgcTa	74	34
hsa-miR-17-3p	acAagTgcmCttmCacTgcAgt	77	47
hsa-miR-17-5p	actAccTgcActGtaAgcActTtg	74	39
hsa-miR-18	tatmCtgmCacTagAtgmCacmCtta	71	40
hsa-miR-181a	acTcamCcgAcaGcgTtgAatGtt	77	49
hsa-miR-181b	cccAccGacAgcAatGaaTgtt	78	30
hsa-miR-181c	amCtcAccGacAggTtgAatGtt	76	33
hsa-miR-182	tgtGagTtcTacmCatTgcmCaaa	72	32
hsa-miR-182*	taGttGgcAagTctAgaAcca	72	32
hsa-miR-183	caGtgAatTctAccAgtGccAta	73	32
hsa-miR-184	acmCctTatmCagTtcTccGtcmCa	76	23
hsa-miR-185	gAacTgcmCttTctmCtcmCa	70	27

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
hsa-miR-186	aaGccmCaaAagGagAatTctTtg	71	48
hsa-miR-187	cGgcTgcAacAcaAgamCacGa	84	31
hsa-miR-188	amCccTccAccAtgmCaaGggAtg	83	42
hsa-miR-189	actGatAtcAgcTcaGtaGgcAc	77	54
hsa-miR-190	acmCtaAtaTatmCaaAcaTatmCa	62	31
hsa-miR-191	agcTgcTttTggGatTccGttg	74	42
hsa-miR-192	gGctGtcAatTcaTagGtcAg	73	46
hsa-miR-193	ctGggActTtgTagGccAgtt	76	31
hsa-miR-194	tccAcaTggAgtTgcTgtTaca	75	41
hsa-miR-195	gmCcaAtaTttmCtgTgcTgcTa	73	28
hsa-miR-196a	ccaAcaAcaTgaAacTacmCta	67	20
hsa-miR-196b	cmCaamCaamCagGaaActAccTa	73	27
hsa-miR-197	gctGggTggAgaAggTggTgaa	84	19
hsa-miR-198	cmCtaTctmCccmCtcTggAcc	75	25
hsa-miR-199a	gaAcaGgtAgtmCtgAacActGgg	78	40
hsa-miR-199a*	aacmCaaTgtGcaGacTacTgta	74	39
hsa-miR-199b	gAacAgaTagTctAaamCacTggg	73	32
hsa-miR-19a	tmCagTttTgcAtaGatTtgmCaca	72	37
hsa-miR-19b	tmCagTttTgcAtgGatTtgmCaca	75	34
hsa-miR-20	ctAccTgcActAtaAgcActTta	70	26
hsa-miR-200a	acaTcgTtamCcaGacAgtGtta	72	39
hsa-miR-200b	gtcAtcAttAccAggmCagTatTa	71	31
hsa-miR-200c	ccAtcAttAccmCggmCagTatTa	74	38
hsa-miR-203	cTagTggTccTaaAcaTttmCac	69	23
hsa-miR-204	aggmCatAggAtgAcaAagGgaa	75	25
hsa-miR-205	caGacTccGgtGgaAtgAagGa	81	39
hsa-miR-206	ccamCacActTccTtamCatTcca	73	11
hsa-miR-208	acAagmCttTttGctmCgtmCttAt	71	34
hsa-miR-21	tmCaamCatmCagTctGatAagmCta	72	48
hsa-miR-210	tcAgcmCgcTgtmCacAcgmCacAg	87	38
hsa-miR-211	aggmCgaAggAtgAcaAagGgaa	77	18
hsa-miR-212	gGccGtgActGgaGacTgtTa	81	37
hsa-miR-213	gGtamCaaTcaAcgGtcGatGgt	79	67
hsa-miR-214	ctGccTgtmCtgTgcmCtgmCtgt	81	30
hsa-miR-215	gtcTgtmCaaTtcAtaGgtmCat	70	50

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
hsa-miR-216	camCagTtgmCcaGctGagAtta	74	64
hsa-miR-217	atcmCaaTcaGttmCctGatGcaGta	75	49
hsa-miR-218	acAtgGttAgaTcaAgcAcaa	70	40
hsa-miR-219	agAatTgcGttTggAcaAtca	70	35
hsa-miR-22	acaGttmCttmCaamCtgGcaGctt	74	48
hsa-miR-220	aaAgtGtcAgaTacGgtGtgg	75	32
hsa-miR-221	gAaamCccAgaAgamCaaTgtAgct	80	31
hsa-miR-222	gaGacmCcaGtaGccAgaTgtAgct	80	38
hsa-miR-223	gGggTatTtgAcaAacTgamCa	73	40
hsa-miR-224	tAaamCggAacmCacTagTgamCttg	75	49
hsa-miR-23a	gGaaAtcmCctGgcAatGtgAt	76	37
hsa-miR-23b	ggtAatmCccTggmCaaTgtGat	76	38
hsa-miR-24	cTgtTccTgcTgaActGagmCca	80	35
hsa-miR-25	tcaGacmCgaGacAagTgcAatg	77	27
hsa-miR-26a	gcmCtaTccTggAttActTgaa	70	34
hsa-miR-26b	aacmCtaTccTgaAttActTgaa	65	28
hsa-miR-27a	gcGgaActTagmCcamCtgTgaa	77	35
hsa-miR-27b	gcAgaActTagmCcamCtgTgaa	74	38
hsa-miR-28	ctmCaaTagActGtgAgaTccTt	73	43
hsa-miR-296	acAggAttGagGggGggmCcct	88	48
hsa-miR-299	aTgtAtgTggGacGgtAaamCca	80	35
hsa-miR-29a	aamCcgAttTcaGatGgtGcta	75	43
hsa-miR-29b	aamCacTgaTttmCaaAtgGtgmCta	71	47
hsa-miR-29c	amCcgAttTcaAatGgtGcta	71	47
hsa-miR-301	gctTtgAcaAtamCtaTtgmCacTg	70	36
hsa-miR-302a	tcAccAaaAcaTggAagmCacTta	72	25
hsa-miR-302a*	aaaGcaAgtAcaTccAcgTtta	69	32
hsa-miR-302b	ctActAaaAcaTggAagmCacTta	69	23
hsa-miR-302b*	agAaaGcamCttmCcaTgtTaaAgt	72	36
hsa-miR-302c	ccActGaaAcaTggAagmCacTta	74	28
hsa-miR-302c*	cagmCagGtamCccmCcaTgtTaaa	76	44
hsa-miR-302d	acActmCaaAcaTggAagmCacTta	73	23
hsa-miR-30a-3p	gctGcaAacAtcmCgamCtgAaag	74	28
hsa-miR-30a-5p	cTtcmCagTcgAggAtgTttAca	73	31
hsa-miR-30b	agcTgaGtgTagGatGttTaca	71	33

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
hsa-miR-30c	gmCtgAgaGtgTagGatGttTaca	73	33
hsa-miR-30d	cttmCcaGtcGggGatGttTaca	76	44
hsa-miR-30e-3p	gmCtgTaaAcaTccGacTgaAag	73	27
hsa-miR-30e-5p	tcmCagTcaAggAtgTttAca	69	30
hsa-miR-31	cagmCtaTgcmCagmCatmCttGcc	78	38
hsa-miR-32	gcaActTagTaaTgtGcaAta	65	43
hsa-miR-320	tTcgmCccTctmCaamCccAgcTtt	80	26
hsa-miR-323	agAggTcgAccGtgTaaTgtGc	80	46
hsa-miR-324-3p	ccAgcAgcAccTggGgcAgtGg	92	41
hsa-miR-324-5p	acAccAatGccmCtaGggGatGcg	84	54
hsa-miR-325	amCacTtamCtgGacAccTacTagg	74	39
hsa-miR-326	ctgGagGaaGggmCccAgaGg	87	46
hsa-miR-328	acGgaAggGcaGagAggGccAg	87	31
hsa-miR-33	cAatGcaActAcaAtgmCac	68	30
hsa-miR-330	tmCtcTgcAgg mCcgTgtGctTtgc	84	53
hsa-miR-331	tTctAggAtaGgcmCcaGggGc	84	51
hsa-miR-335	amCatTttTcgTtaTtgmCtcTtga	67	26
hsa-miR-337	aaaGgcAtcAtaTagGagmCtgGa	76	34
hsa-miR-338	tcaAcaAaaTcamCtgAtgmCtgGa	73	33
hsa-miR-339	tgAgcTccTggAggAcaGgga	83	47
hsa-miR-340	ggcTatAaaGtaActGagAcgGa	72	34
hsa-miR-342	gacGggTgcGatTtcTgtGtgAga	82	34
hsa-miR-345	gmCccTggActAggAgtmCagmCa	84	40
hsa-miR-346	agaGgcAggmCatGcgGgcAgamCa	92	50
hsa-miR-34a	aamCaamCcaGctAagAcamCtgmCca	80	27
hsa-miR-34b	cAatmCagmCtaAtgAcamCtgmCcta	74	30
hsa-miR-34c	gcAatmCagmCtaActAcamCtgmCct	76	31
hsa-miR-361	gTacmCccTggAgaTtcTgaTaa	73	29
hsa-miR-367	tmCacmCatTgcTaaAgtGcaAtt	72	41
hsa-miR-368	aaamCgtGgaAttTccTctAtgt	70	45
hsa-miR-369	aaAgaTcaAccAtgTatTatt	62	24
hsa-miR-370	ccAggTtcmCacmCccAgcAggc	86	29
hsa-miR-371	acActmCaaAagAtgGcgGcac	76	33
hsa-miR-372	acgmCtcAaaTgtmCgcAgtActTt	79	38
hsa-miR-373	acamCccmCaaAatmCgaAgtActTc	77	33

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
hsa-miR-373*	ggaAagmCgcmCccmCatTttGagt	78	31
hsa-miR-374	cacTtaTcaGgtTgtAttAtaa	63	35
hsa-miR-375	tcAcgmCgaGccGaamCgaAcaAa	81	39
hsa-miR-376a	acgTggAttTtcmCtcTatGat	68	39
hsa-miR-377	acAaaAgtTgcmCttTgtGtgAt	73	48
hsa-miR-378	amCacAggAccTggAgtmCagGag	84	51
hsa-miR-379	tacGttmCcaTagTctAcca	66	25
hsa-miR-380-3p	aagAtgTggAccAtaTtamCata	66	54
hsa-miR-380-5p	gmCgcAtgTtcTatGgtmCaamCca	80	41
hsa-miR-381	amCagAgaGctTgcmCctTgtAta	76	37
hsa-miR-382	cgaAtcmCacmCacGaamCaamCttc	75	23
hsa-miR-383	agcmCacAatmCacmCttmCtgAtct	74	27
hsa-miR-384	tAtgAacAatTtcTagGaat	61	46
hsa-miR-422a	ggcmCttmCtgAccmCtaAgtmCcag	76	45
hsa-miR-422b	ggmCctTctGacTccAagTccAg	80	39
hsa-miR-423	ctgAggGgcmCtcAgamCcgAgct	87	61
hsa-miR-424	ttcAaaAcaTgaAttGctGctg	69	40
hsa-miR-425	ggmCggAcamCgamCatTccmCgat	83	43
hsa-miR-7	caamCaaAatmCacTagTctTcca	69	30
hsa-miR-9	tcAtamCagmCtaGatAacmCaaAga	71	34
hsa-miR-9*	acTttmCggTtaTctAgtTta	65	27
hsa-miR-92	cagGccGggAcaAgtGcaAta	79	28
hsa-miR-93	ctAccTgcAcgAacAgcActTt	76	31
hsa-miR-95	tgcTcaAtaAatAccmCgtTgaa	68	36
hsa-miR-96	gcaAaaAtgTgcTagTgcmCaaa	72	38
hsa-miR-98	aAcaAtamCaamCttActAccTca	67	17
hsa-miR-99a	cacAagAtcGgaTctAgtGgtt	77	42
hsa-miR-99b	cgcAagGtcGgtTctAgtGgtg	82	42
mmu-let-7a	amCtaTacAacmCtamCtamCctmCa	71	16
mmu-let-7b	aamCcamCacAacmCtamCtamCctmCa	77	6
mmu-let-7c	aamCcaTacAacmCtamCtamCctmCa	74	11
mmu-let-7d	actAtgmCaamCctActAccTct	71	24
mmu-let-7d*	agAaaGgcAgtGggTcgTatAg	79	23
mmu-let-7e	actAtamCaamCctmCctAccTca	71	16
mmu-let-7f	amCtaTacAatmCtamCtamCctmCa	68	16

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
mmu-let-7g	amCtgTacAaamCtamCtamCctmCa	71	30
mmu-let-7i	amCagmCacAaamCtamCtamCctmCa	76	18
mmu-miR-1	tamCatActTctTtamCatTcca	64	11
mmu-miR-100	cacAagTtcGgaTctAcgGgtt	77	38
mmu-miR-101a	cttmCagTtaTcamCagTacTgta	68	54
mmu-miR-101b	cttmCagmCtaTcamCagTacTgta	70	54
mmu-miR-103	tmCatAgcmCctGtamCaaTgcTgct	80	63
mmu-miR-106a	tacmCtgmCacTgtTagmCacTttg	73	44
mmu-miR-106b	atcTgcActGtcAgcActTta	72	35
mmu-miR-107	tGatAgcmCctGtamCaaTgcTgct	80	63
mmu-miR-10a	cAcaAatTcgGatmCtamCagGgta	74	37
mmu-miR-10b	acamCaaAttmCggTtcTacAggg	73	27
mmu-miR-122a	acAaamCacmCatTgtmCacActmCca	78	25
mmu-miR-124a	ggmCatTcamCcgmCgtGccTta	80	43
mmu-miR-125a	cAcaGgtTaaAggGtcTcaGgga	79	35
mmu-miR-125b	tcamCaaGttAggGtcTcaGgga	77	35
mmu-miR-126-3p	gcAttAttActmCacGgtAcga	71	25
mmu-miR-126-5p	cgmCgtAccAaaAgtAatAatg	68	28
mmu-miR-127	gcmCaaGctmCagAcgGatmCcga	80	54
mmu-miR-128a	aaAagAgamCcgGttmCacTgtGa	77	47
mmu-miR-128b	gaAagAgamCcgGttmCacTgtGa	78	47
mmu-miR-129	agcAagmCccAgamCcgmCaaAaag	80	21
mmu-miR-129-3p	aTgcTttTtgGggTaaGggmCtt	78	37
mmu-miR-129-5p	agcAagmCccAgamCcgmCaaAaag	80	21
mmu-miR-130a	aTgcmCctTttAacAttGcamCtg	74	42
mmu-miR-130b	aTgcmCctTtcAtcAttGcamCtg	75	34
mmu-miR-132	cgAccAtgGctGtaGacTgtTa	76	48
mmu-miR-133a	acAgcTggTtgAagGggAccAa	82	41
mmu-miR-133b	taGctGgtTgaAggGgamCcaa	81	37
mmu-miR-134	cccmCtcTggTcaAccAgtmCaca	79	57
mmu-miR-135a	tcamCatAggAatAaaAagmCcaTa	69	22
mmu-miR-135b	cacAtaGgaAtgAaaAgcmCata	70	22
mmu-miR-136	tccAtcAtcAaaAcaAatGgaGt	71	25
mmu-miR-137	cTacGcgTatTctTaaGcaAtaa	67	48
mmu-miR-138	gatTcamCaamCacmCagmCt	70	24

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
mmu-miR-139	aGacAcgTgcActGtaGa	75	42
mmu-miR-140	ctamCcaTagGgtAaaAccActg	71	56
mmu-miR-140*	tcmCgtGgtTctAccmCtgTggTa	81	49
mmu-miR-141	cmCatmCttTacmCagAcaGtgTta	70	33
mmu-miR-142-3p	ccaTaaAgtAggAaamCacTaca	69	29
mmu-miR-142-5p	gtaGtgmCttTctActTtaTg	63	36
mmu-miR-143	tGagmCtamCagTgcTtcAtcTca	75	56
mmu-miR-144	ctaGtamCatmCatmCtaTacTgta	64	37
mmu-miR-145	aAggGatTccTggGaaAacTggAc	79	50
mmu-miR-146	aAccmCatGgaAttmCagTtcTca	73	44
mmu-miR-148a	acaAagTtcTgtAgtGcamCtga	72	54
mmu-miR-148b	acaAagTtcTgtGatGcamCtga	72	39
mmu-miR-149	ggaGtgAagAcamCggAgcmCaga	80	31
mmu-miR-150	cacTggTacAagGgtTggGaga	78	30
mmu-miR-151	cmCtcAagGagmCctmCagTctAg	78	42
mmu-miR-152	cmCcaAgtTctGtcAtgmCacTga	78	36
mmu-miR-153	gatmCacTttTgtGacTatGcaa	69	36
mmu-miR-154	cGaaGgcAacAcgGatAacmCta	78	40
mmu-miR-155	ccmCctAtcAcaAttAgtAttAa	69	21
mmu-miR-15a	cAcaAacmCatTatGtgmCtgmCta	73	35
mmu-miR-15b	tgtAaamCcaTgaTgtGctGcta	74	38
mmu-miR-16	cgmCcaAtaTttAcgTgcTgcTa	74	34
mmu-miR-17-3p	tacAagTgcmCctmCacTgcAgt	79	42
mmu-miR-17-5p	actAccTgcActGtaAgtActTtg	74	39
mmu-miR-18	tatmCtgmCacTagAtgmCacmCtta	71	40
mmu-miR-181a	acTcamCcgAcaGcgTtgAatGtt	77	49
mmu-miR-181b	cccAccGacAgtAatGaaTgtt	78	30
mmu-miR-181c	amCtcAccGacAgtTtgAatGtt	76	33
mmu-miR-182	tgtGagTtcTacmCatTgcmCaaa	72	32
mmu-miR-183	caGtgAatTctAccAgtGccAta	73	32
mmu-miR-184	acmCctTatmCagTtcTccGtcmCa	76	23
mmu-miR-185	gAacTgcmCttTctmCtcmCa	70	27
mmu-miR-186	aaGccmCaaAagGagAatTctTtg	71	48
mmu-miR-187	ccGgcTgcAacAcaAgamCacGa	85	31
mmu-miR-188	amCccTccAccAtgmCaaGggAtg	83	42

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
mmu-miR-189	actGatAtcAgcTcaGtaGgcAc	77	54
mmu-miR-190	acmCtaAtaTatmCaaAcaTatmCa	62	31
mmu-miR-191	agcTgcTttTggGatTccGttg	74	42
mmu-miR-192	tgTcaAttmCatAggTcag	64	28
mmu-miR-193	ctGggActTtgTagGccAgtt	76	31
mmu-miR-194	tccAcaTggAgtTgcTgtTaca	75	41
mmu-miR-195	gmCcaAtaTttmCtgTgcTgcTa	73	28
mmu-miR-196a	ccaAcaAcaTgaAacTacmCta	67	20
mmu-miR-196b	cmCaamCaamCagGaaActAccTa	73	27
mmu-miR-199a	gaAcaGgtAgtmCtgAacActGgg	78	40
mmu-miR-199a*	aacmCaaTgtGcaGacTacTgta	74	39
mmu-miR-199b	gaAcaGgtAgtmCtaAacActGgg	76	31
mmu-miR-19a	tmCagTttTgcAtaGatTtgmCaca	72	37
mmu-miR-19b	tmCagTttTgcAtgGatTtgmCaca	75	34
mmu-miR-20	ctAccTgcActAtaAgcActTta	70	26
mmu-miR-200a	acaTcgTtamCcaGacAgtGtta	72	39
mmu-miR-200b	gtcAtcAttAccAggmCagTatTa	71	31
mmu-miR-200c	ccAtcAttAccmCggmCagTatTa	74	38
mmu-miR-201	agAacAatGccTtamCtgAgta	69	37
mmu-miR-202	tmCttmCccAtgmCgcTatAccTct	76	28
mmu-miR-203	cTagTggTccTaaAcaTttmCa	68	23
mmu-miR-204	cagGcaTagGatGacAaaGggAa	78	25
mmu-miR-205	caGacTccGgtGgaAtgAagGa	81	39
mmu-miR-206	ccamCacActTccTtamCatTcca	73	11
mmu-miR-207	gaGggAggAgaGccAggAgaAgc	86	18
mmu-miR-208	acAagmCttTttGctmCgtmCttAt	71	34
mmu-miR-21	tmCaamCatmCagTctGatAagmCta	72	48
mmu-miR-210	tcAgcmCgcTgtmCacAcgmCacAg	87	38
mmu-miR-211	aggmCaaAggAtgAcaAagGgaa	75	18
mmu-miR-212	gGccGtgActGgaGacTgtTa	81	37
mmu-miR-213	gGtamCaaTcaAcgGtcGatGgt	79	67
mmu-miR-214	ctGccTgtmCtgTgcmCtgmCtgt	81	30
mmu-miR-215	gtmCtgTcaAatmCatAggTcat	68	35
mmu-miR-216	camCagTtgmCcaGctGagAtta	74	64
mmu-miR-217	atmCcaGtcAgtTccTgaTgcAgta	77	43

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
mmu-miR-218	acAtgGttAgaTcaAgaAcaa	70	40
mmu-miR-219	agAatTgcGttTggAcaAtca	70	35
mmu-miR-22	acaGttmCttmCaamCtgGcaGctt	74	48
mmu-miR-221	aaamCccAgaAgamCaaTgtAgct	79	31
mmu-miR-222	gaGacmCcaGtaGccAgaTgtAgct	80	38
mmu-miR-223	gGggTatTtgAcaAacTgamCa	73	40
mmu-miR-224	tAaamCggAacmCacTagTgamCtta	74	49
mmu-miR-23a	gGaaAtcmCctGgcAatGtgAt	76	37
mmu-miR-23b	ggtAatmCccTggmCaaTgtGat	76	38
mmu-miR-24	cTgtTccTgcTgaActGagmCca	80	35
mmu-miR-25	tcaGacmCgaGacAagTgcAatg	77	27
mmu-miR-26a	gcmCtaTccTggAttActTgaa	70	34
mmu-miR-26b	aacmCtaTccTgaAttActTgaa	65	28
mmu-miR-27a	gcGgaActTagmCcamCtgTgaa	77	35
mmu-miR-27b	gcAgaActTagmCcamCtgTgaa	74	38
mmu-miR-28	ctmCaaTagActGtgAgaTccTt	73	43
mmu-miR-290	aaaAagTgcmCccmCatAgtTtgAg	75	29
mmu-miR-291-3p	gGcamCacAaaGtgGaaGcamCtt	78	52
mmu-miR-291-5p	aGagAggGccTccActTtgAtg	77	46
mmu-miR-292-3p	acActmCaaAacmCtgGcgGcamCtt	80	33
mmu-miR-292-5p	caaAagAgcmCccmCagTttGagt	76	32
mmu-miR-293	amCacTacAaamCtcTgcGgcAct	81	30
mmu-miR-294	acAcamCaaAagGgaAgaCtTt	75	25
mmu-miR-295	agamCtcAaaAgtAgtAgaCtTt	70	44
mmu-miR-296	acAggAttGagGggGggmCcct	88	48
mmu-miR-297	cAtgmCacAtgmCacAcaTacAt	75	41
mmu-miR-298	gGaaGaamCagmCccTccTctGcc	82	53
mmu-miR-299	aTgtAtgTggGacGgtAaamCca	80	35
mmu-miR-29a	aamCcgAttTcaGatGgtGcta	75	43
mmu-miR-29b	aamCacTgaTttmCaaAtgGtgmCta	71	47
mmu-miR-29c	amCcgAttTcaAatGgtGcta	71	47
mmu-miR-300	gAagAgaGctTgcmCctTgcAta	77	35
mmu-miR-301	gctTtgAcaAtamCtaTtgmCacTg	70	36
mmu-miR-302	tcAccAaaAcaTggAagmCacTta	72	25
mmu-miR-30a-3p	gctGcaAacAtcmCgamCtgAaag	74	28

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-compli- ment. score
mmu-miR-30a-5p	cTtcnCagTcgAggAtgTttAca	73	31
mmu-miR-30b	agcTgaGtgTagGatGttTaca	71	33
mmu-miR-30c	gmCtgAgaGtgTagGatGttTaca	73	33
mmu-miR-30d	cttmCcaGtcGggGatGttTaca	76	44
mmu-miR-30e	tcmCagTcaAggAtgTttAca	69	30
mmu-miR-30e*	ctgTaaAcaTccGacTgaAag	69	27
mmu-miR-31	cagmCtaTgcmCagmCatmCttGcct	79	38
mmu-miR-32	gcaActTagTaaTgtGcaAta	65	43
mmu-miR-320	tTcgmCccTctmCaamCccAgcTtt	80	26
mmu-miR-322-3p	tgtTgcAgcGctTcaTgtTt	74	48
mmu-miR-322-5p	tccAaaAcaTgaAttGctGctg	71	40
mmu-miR-323	agAggTcgAccGtgTaaTgtGc	80	46
mmu-miR-324-3p	ccAgcAgcAccTggGgcAgtGg	92	41
mmu-miR-324-5p	cAccAatGccmCtaGggGatGcg	83	54
mmu-miR-325	acamCttActGagmCacmCtamCtaGg	78	42
mmu-miR-326	actGgaGgaAggGccmCagAgg	86	46
mmu-miR-328	acGgaAggGcaGagAggGccAg	87	31
mmu-miR-329	aAaaAggTtaGctGggTgtGtt	75	32
mmu-miR-33	cAatGcaActAcaAtgmCac	68	30
mmu-miR-330	tmCtcTgcAggmCccTgtGctTtgc	83	52
mmu-miR-331	tTctAggAtaGgcmCcaGggGc	84	51
mmu-miR-335	amCatTttTcgTtaTtgmCtcTtga	67	26
mmu-miR-337	aaaGgcAtcAtaTagGagmCtgAa	74	35
mmu-miR-338	tcaAcaAaaTcamCtgAtgmCtgGa	73	33
mmu-miR-339	tgAgcTccTggAggAcaGgga	83	47
mmu-miR-340	ggcTatAaaGtaActGagAcgGa	72	34
mmu-miR-341	amCtgAccGacmCgamCcgAtcGa	84	53
mmu-miR-342	gacGggTgcGatTtcTgtGtgAga	82	34
mmu-miR-344	amCagTcaGgcTttGgcTagAtca	79	53
mmu-miR-345	gcActGgammCtaGggGtcAgca	83	43
mmu-miR-346	agaGgcAggmCacTcgGgcAgamCa	91	38
mmu-miR-34a	aamCaamCcaGctAagAcamCtgmCca	80	27
mmu-miR-34b	caaTcaGctAatTacActGccTa	71	40
mmu-miR-34c	gcAatmCagmCtaActAcamCtgmCct	76	31
mmu-miR-350	tgaAagTgtAtgGgcTttGtgAa	73	42

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
mmu-miR-351	cagGctmCaaAggGctmCctmCagGga	84	59
mmu-miR-361	gTacmCccTggAgaTtcTgaTaa	73	29
mmu-miR-370	aAccAggTtcmCacmCccAgcAggc	86	34
mmu-miR-375	tcAcgmCgaGccGaamCgaAcaAa	81	39
mmu-miR-376a	acgTggAttTtcmCtcTacGat	71	47
mmu-miR-376b	aAagTggAtgTtcmCtcTatGat	70	39
mmu-miR-377	acAaaAgtTgcmCttTgtGtgAt	73	48
mmu-miR-378	amCacAggAccTggAgtmCagGag	84	51
mmu-miR-379	cctAcgTtcmCatAgtmCtamCca	72	33
mmu-miR-380-3p	aagAtgTggAccAtamCtamCata	69	49
mmu-miR-380-5p	gmCgcAtgTtcTatGgtmCaamCca	80	41
mmu-miR-381	amCagAgaGctTgcmCctTgtAta	76	37
mmu-miR-382	cgaAtcmCacmCacGaamCaamCttc	75	23
mmu-miR-383	agcmCacAgtmCacmCttmCtgAtct	76	25
mmu-miR-384	tGtgAacAatTtcTagGaat	64	46
mmu-miR-409	aAggGgtTcamCcgAgcAacAttc	80	35
mmu-miR-410	aacAggmCcaTctGtgTtaTatt	70	39
mmu-miR-411	actGagGgtTagTggAccGtgTt	80	40
mmu-miR-412	acgGctAgtGgammCcaGgtGaaGt	86	53
mmu-miR-425	ggmCggAcamCgamCatTccmCgat	83	43
mmu-miR-7	caamCaaAatmCacTagTctTcca	69	30
mmu-miR-7b	aamCaaAatmCacAagTctTcca	68	24
mmu-miR-9	cAtamCagmCtaGatAacmCaaAga	70	34
mmu-miR-9*	acTttmCggTtaTctAgtTta	65	27
mmu-miR-92	cagGccGggAcaAgtGcaAta	79	28
mmu-miR-93	ctAccTgcAcgAacAgtActTtg	77	31
mmu-miR-96	aGcaAaaAtgTgcTagTgcmCaaa	75	38
mmu-miR-98	aAcaAtamCaamCttActAccTca	67	17
mmu-miR-99a	acAagAtcGgaTctAgtGgt	77	40
mmu-miR-99b	cgcAagGtcGgtTctAgtGgt	82	42
osa-miR156a	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156b	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156c	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156d	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156e	gtgmCtcActmCtcTtcTgtmCa	71	25

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
osa-miR156f	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156g	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156h	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156i	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156j	gtgmCtcActmCtcTtcTgtmCa	71	25
osa-miR156k	tgTgcTctmCtcTctTctGtca	72	21
osa-miR156l	taTgcTcamCtcTctTctGtgc	71	17
osa-miR159a	cagAgcTccmCttmCaaTccAaa	73	36
osa-miR159b	cagAgcTccmCttmCaaTccAaa	73	36
osa-miR159c	tggAgcTccmCttmCaaTccAat	74	46
osa-miR159d	cggAgcTccmCttmCaaTccAat	75	46
osa-miR159e	aggAgcTccmCttmCaaTccAat	74	46
osa-miR159f	tagAgcTccmCttmCaaTccAag	72	36
osa-miR160a	tggmCatAcaGggAgcmCagGca	85	49
osa-miR160b	tggmCatAcaGggAgcmCagGca	85	49
osa-miR160c	tggmCatAcaGggAgcmCagGca	85	49
osa-miR160d	tggmCatAcaGggAgcmCagGca	85	49
osa-miR160e	cggmCatAcaGggAgcmCagGca	85	43
osa-miR160f	tgGcaTtcAggGagmCcaGgca	84	60
osa-miR162a	ctgGatGcaGagGttTatmCga	73	34
osa-miR162b	ctgGatGcaGagGctTatmCga	76	36
osa-miR164a	tgcAcgTgcmCctGctTctmCca	82	46
osa-miR164b	tgcAcgTgcmCctGctTctmCca	82	46
osa-miR164c	tGcamCgtAccmCtgmCttmCtcmCa	82	32
osa-miR164d	agcAcgTgcmCctGctTctmCca	82	47
osa-miR164e	ctcAcgTgcmCctGctTctmCca	80	36
osa-miR166a	gGggAatGaaGccTggTccGa	84	33
osa-miR166b	gGggAatGaaGccTggTccGa	84	33
osa-miR166c	gGggAatGaaGccTggTccGa	84	33
osa-miR166d	gGggAatGaaGccTggTccGa	84	33
osa-miR166e	gGggAatGaaGccTggTccGa	84	33
osa-miR166f	gGggAatGaaGccTggTccGa	84	33
osa-miR166g	gAggAatGaaGccTggTccGa	80	29
osa-miR166h	gAggAatGaaGccTggTccGa	80	29
osa-miR166i	gAggAatGaaGccTgaTccGa	78	29

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
osa-miR166j	gAgAAatGaaGccTgaTccGa	78	29
osa-miR166k	aGggAttGaaGccTggTccGa	83	37
osa-miR166l	aGggAttGaaGccTggTccGa	83	37
osa-miR167a	tAgaTcaTgcTggmCagmCttmCa	79	53
osa-miR167b	tAgaTcaTgcTggmCagmCttmCa	79	53
osa-miR167c	tAgaTcaTgcTggmCagmCttmCa	79	53
osa-miR167d	cAgaTcaTgcTggmCagmCttmCa	80	53
osa-miR167e	cAgaTcaTgcTggmCagmCttmCa	80	53
osa-miR167f	cAgaTcaTgcTggmCagmCttmCa	80	53
osa-miR167g	cAgaTcaTgcTggmCagmCttmCa	80	53
osa-miR167h	cAgaTcaTgcTggmCagmCttmCa	80	53
osa-miR167i	cAgaTcaTgcTggmCagmCttmCa	80	53
osa-miR168a	gtmCccGatmCtgmCacmCaaGcga	82	38
osa-miR168b	ttcmCcgAgcTgcAccAagmCct	83	30
osa-miR169a	tcGgcAagTcaTccTtgGctg	78	40
osa-miR169b	ccGgcAagTcaTccTtgGctg	79	40
osa-miR169c	ccGgcAagTcaTccTtgGctg	79	40
osa-miR169d	ccGgcAatTcaTccTtgGcta	76	33
osa-miR169e	ccGgcAagTcaTccTtgGcta	78	35
osa-miR169f	taGgcAagTcaTccTtgGcta	74	47
osa-miR169g	taGgcAagTcaTccTtgGcta	74	47
osa-miR169h	caGgcAagTcaTccTtgGcta	76	41
osa-miR169i	caGgcAagTcaTccTtgGcta	76	41
osa-miR169j	caGgcAagTcaTccTtgGcta	76	41
osa-miR169k	caGgcAagTcaTccTtgGcta	76	41
osa-miR169l	caGgcAagTcaTccTtgGcta	76	41
osa-miR169m	caGgcAagTcaTccTtgGcta	76	41
osa-miR169n	taGgcAagTcaTtcTtgGcta	71	47
osa-miR169o	taGgcAagTcaTtcTtgGcta	71	47
osa-miR169p	ccgGcaAgtTtgTccTtgGcta	76	52
osa-miR169q	caTggGcaGtcTccTtgGcta	75	47
osa-miR171a	gAtaTtgGcgmCggmCtcAatmCa	78	54
osa-miR171b	gaTatTggmCacGgcTcaAtca	75	46
osa-miR171c	gaTatTggmCacGgcTcaAtca	75	46
osa-miR171d	gaTatTggmCacGgcTcaAtca	75	46

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
osa-miR171e	gaTatTggmCacGgcTcaAtca	75	46
osa-miR171f	gaTatTggmCacGgcTcaAtca	75	46
osa-miR171g	gaTatTggmCtcGgcTcamCctc	78	34
osa-miR171h	agTgaTatTggTtcGgcTcac	74	34
osa-miR172a	atgmCagmCatmCatmCaaGatTct	73	45
osa-miR172b	aTgcAgcAtcAtcAagAttmCc	74	39
osa-miR172c	gTgcAgcAtcAtcAagAttmCa	74	39
osa-miR319a	gggAgcAccmCttmCagTccAa	78	39
osa-miR319b	gggAgcAccmCttmCagTccAa	78	39
osa-miR393	gAtcAatGcgAtcmCctTtgGa	74	56
osa-miR393b	agaTcaAtgmCgaTccmCttTgga	73	56
osa-miR394	gGagGtgGacAgaAtgmCcaa	77	29
osa-miR395a	gagTtcmCccmCaaAtamCttmCac	71	23
osa-miR395b	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395c	gAgtTccmCccAaamCacTtcAc	78	28
osa-miR395d	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395e	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395f	gatTtcmCccmCaaAcgmCttmCac	74	22
osa-miR395g	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395h	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395i	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395j	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395k	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395l	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395m	gAgtTccmCccAaamCacTtcAc	75	28
osa-miR395n	gAgtTtcmCccAaamCacTtcAc	73	35
osa-miR395o	gAgtTtcmCccAaamCacTtcAc	73	35
osa-miR395p	gatTtcmCccmCaaAcgmCttmCac	74	22
osa-miR395q	gAgtTccTccAaamCacTtcAc	72	29
osa-miR395r	gAgtTtcmCccAaamCacTtcAc	73	35
osa-miR395s	gatTtcmCccmCaaAcgmCttmCac	74	22
osa-miR396a	cagTtcAagAaaGctGtgGaa	70	35
osa-miR396b	cagTtcAagAaaGctGtgGaa	70	35
osa-miR396c	aagTtcAagAaaGctGtgGaa	69	24
osa-miR397a	caTcaAcgmCtgmCacTcaAtga	73	39

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
osa-miR397b	caTcaAcgmCtgmCacTcaAtaa	71	35
osa-miR398a	aagGggTgamCctGagAacAca	80	39
osa-miR398b	caGggGcgAccTgaGaamCaca	83	43
osa-miR399a	cAggGcaAttmCtcmCttTggmCa	78	48
osa-miR399b	cAggGcaAttmCtcmCttTggmCa	78	48
osa-miR399c	cAggGcaAttmCtcmCttTggmCa	78	48
osa-miR399d	caGggmCaamCtcTccTttGgca	81	39
osa-miR399e	cTggGcaAatmCtcmCttTggmCa	77	41
osa-miR399f	cTggGcaAatmCtcmCttTggmCa	77	41
osa-miR399g	cmCggGcaAatmCtcmCttTggmCa	80	41
osa-miR399h	cTggGcaAgtmCtcmCttTggmCa	80	37
osa-miR399i	caGggmCagmCtcTccTttGgca	83	63
osa-miR399j	taGggmCaamCtcTccTttGgca	80	39
osa-miR399k	cggGgcAaaTttmCctTtgGca	76	53
osa-miR408	gmCcaGggAagAggmCagTgcAg	88	35
osa-miR413	gtgmCagAacAagTgaAacTag	70	24
osa-miR414	gGacGatGatGatGagGatGa	77	21
osa-miR415	ctgmCtcTgcTtcTgtTctGtt	71	19
osa-miR416	tgAacAgtGtamCggAcgAaca	75	42
osa-miR417	tgGaamCaaAttmCacTacAttc	66	26
osa-miR418	cgTcaTttmCatmCatmCacAtta	67	16
osa-miR419	caamCatmCgtmCagmCatTcaTca	74	18
osa-miR420	atcAttTccGtgAttAatTta	60	32
osa-miR426	cgtAagGacAaamCttmCcaAaa	69	31
rno-let-7a	aamCtaTacAacmCtamCtamCctmCa	70	16
rno-let-7b	aamCcamCacAacmCtamCtamCctmCa	77	6
rno-let-7c	aamCcaTacAacmCtamCtamCctmCa	74	11
rno-let-7d	actAtgmCaamCctActAccTct	71	24
rno-let-7d*	agAaaGgcAgcAggTcgTatAg	79	23
rno-let-7e	actAtamCaamCctmCctAccTca	71	16
rno-let-7f	aamCtaTacAatmCtamCtamCctmCa	67	16
rno-let-7i	amCagmCacAaamCtamCtamCctmCa	76	18
rno-miR-100	cacAagTtcGgaTctAcgGtt	77	38
rno-miR-101	cttmCagTtaTcamCagTacTgta	68	54
rno-miR-101b	cttmCagmCtaTcamCagTacTgta	70	54

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
rno-miR-103	trnCatAgcmCctGtamCaaTgcTgct	80	63
rno-miR-106b	atcTgcActGtcAgcActTta	72	35
rno-miR-107	tGatAgcmCctGtamCaaTgcTgct	80	63
rno-miR-10a	cAcaAatTcgGatmCtamCagGgta	74	37
rno-miR-10b	acamCaaAttmCggTtcTacAggg	73	27
rno-miR-122a	acAaamCacmCatTgtmCacActmCca	78	25
rno-miR-124a	tggmCatTcamCcgmCgtGccTtaa	80	43
rno-miR-125a	cAcaGgtTaaAggGtcTcaGgga	79	35
rno-miR-125b	tcamCaaGttAggGtcTcaGgga	77	35
rno-miR-126	gcAttAttActmCacGgtAcga	71	25
rno-miR-126*	cgmCgtAccAaaAgtAatAatg	68	28
rno-miR-127	agcmCaaGctmCagAcgGatmCcga	81	54
rno-miR-128a	aaAagAgamCcgGttmCacTgtGa	77	47
rno-miR-128b	gaAagAgamCcgGttmCacTgtGa	78	47
rno-miR-129	agcAagmCccAgamCcgmCaaAaag	80	21
rno-miR-129*	aTgcTttTtgGggTaaGggmCtt	78	37
rno-miR-130a	aTgcmCctTttAacAttGcamCtg	74	42
rno-miR-130b	aTgcmCctTtcAtcAttGcamCtg	75	34
rno-miR-132	cgAccAtgGctGtaGacTgtTa	76	48
rno-miR-133a	acAgcTggTtgAagGggAccAa	82	41
rno-miR-134	ccmCtcTggTcaAccAgtmCaca	77	57
rno-miR-135a	tcamCatAggAatAaaAagmCcaTa	69	22
rno-miR-135b	cacAtaGgaAtgAaaAgcmCata	70	22
rno-miR-136	tccAtcAtcAaaAcaAatGgaGt	71	25
rno-miR-137	cTacGcgTatTctTaaGcaAta	68	48
rno-miR-138	gatTcamCaamCacmCagmCt	70	24
rno-miR-139	aGacAcgTgcActGtaGa	75	42
rno-miR-140	ctAccAtaGggTaaAacmCact	71	43
rno-miR-140*	tgtmCcgTggTtcTacmCctGtgGta	80	50
rno-miR-141	cmCatmCttTacmCagAcaGtgTta	70	33
rno-miR-142-3p	tmCcaTaaAgtAggAaamCacTaca	72	29
rno-miR-142-5p	gtaGtgmCttTctActTtaTg	63	36
rno-miR-143	tGagmCtamCagTgcTtcAtcTca	75	56
rno-miR-144	ctaGtamCatmCatmCtaTacTgta	64	37
rno-miR-145	aAggGatTccTggGaaAacTggAc	79	50

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
rno-miR-146	aAccmCatGgaAttmCagTtcTca	73	44
rno-miR-148b	acaAagTtcTgtGatGcamCtga	72	39
rno-miR-150	cacTggTacAagGgtTggGaga	78	30
rno-miR-151	cmCtcAagGagmCctmCagTctAgt	78	42
rno-miR-151*	tacTagActGtgAgcTccTcga	74	42
rno-miR-152	cmCcaAgtTctGtcAtgmCacTga	78	36
rno-miR-153	tcamCttTtgTgamCtaTgcAa	68	35
rno-miR-154	cGaaGgcAacAcgGatAacmCta	78	40
rno-miR-15b	tgtAaamCcaTgaTgtGctGcta	74	38
rno-miR-16	cgmCcaAtaTttAcgTgcTgcTa	74	34
rno-miR-17	actAccTgcActGtaAgcActTtg	74	39
rno-miR-18	tatmCtgmCacTagAtgmCacmCtta	71	40
rno-miR-181a	acTcamCcgAcaGcgTtgAatGtt	77	49
rno-miR-181b	cccAccGacAgcAatGaaTgtt	78	30
rno-miR-181c	amCtcAccGacAggTtgAatGtt	76	33
rno-miR-183	caGtgAatTctAccAgtGccAta	73	32
rno-miR-184	acmCctTatmCagTtcTccGtcmCa	76	23
rno-miR-185	gAacTgcmCttTctmCtcmCa	70	27
rno-miR-186	aaGccmCaaAagGagAatTctTtg	71	48
rno-miR-187	cGgcTgcAacAcaAgamCacGa	84	31
rno-miR-190	acmCtaAtaTatmCaaAcaTatmCa	62	31
rno-miR-191	agcTgcTttTggGatTccGttg	74	42
rno-miR-192	gGctGtcAatTcaTagGtcAg	73	46
rno-miR-193	ctGggActTtgTagGccAgtt	76	31
rno-miR-194	tccAcaTggAgtTgcTgtTaca	75	41
rno-miR-195	gmCcaAtaTttmCtgTgcTgcTa	73	28
rno-miR-196a	ccaAcaAcaTgaAacTacmCta	67	20
rno-miR-196b	cmCaamCaamCagGaaActAccTa	73	27
rno-miR-199a	gaAcaGgtAgtmCtgAacActGgg	78	40
rno-miR-19a	tmCagTttTgcAtaGatTtgmCaca	72	37
rno-miR-19b	tmCagTttTgcAtgGatTtgmCaca	75	34
rno-miR-20	ctAccTgcActAtaAgcActTta	70	26
rno-miR-20*	tgtAagTgcTcgTaaTgcAgt	74	26
rno-miR-200a	acaTcgTtamCcaGacAgtGtta	72	39
rno-miR-200b	gtcAtcAttAccAggmCagTatTa	71	31

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-compl. score
rno-miR-200c	ccAtcAttAccmCggmCagTatTa	74	38
rno-miR-203	cTagTggTccTaaAcaTttmCac	69	23
rno-miR-204	aggmCatAggAtgAcaAagGgaa	75	25
rno-miR-205	caGacTccGgtGgaAtgAagGa	81	39
rno-miR-206	ccamCacActTccTttaCatTcca	73	11
rno-miR-208	acAagmCttTttGctmCgtmCttAt	71	34
rno-miR-21	tmCaamCatmCagTctGatAagmCta	72	48
rno-miR-210	tcAgcmCgcTgtmCacAcgmCacAg	87	38
rno-miR-211	aggmCaaAggAtgAcaAagGgaa	75	18
rno-miR-212	gGccGtgActGgaGacTgtTa	81	37
rno-miR-213	gGtamCaaTcaAcgGtcGatGgt	79	67
rno-miR-214	ctGccTgtmCtgTgcmCtgmCtgt	81	30
rno-miR-216	camCagTtgmCcaGctGagAtta	74	64
rno-miR-217	atmCcaGtcAgtTccTgaTgcAgta	77	43
rno-miR-218	acAtgGttAgaTcaAgcAcaa	70	40
rno-miR-219	agAatTgcGttTggAcaAtca	70	35
rno-miR-22	acaGttmCttmCaamCtgGcaGctt	74	48
rno-miR-221	gAaamCccAgcAgamCaaTgtAgct	80	31
rno-miR-222	gaGacmCcaGtaGccAgaTgtAgct	80	38
rno-miR-223	gGggTatTtgAcaAacTgamCa	73	40
rno-miR-23a	gGaaAtcmCctGgcAatTtgAt	76	37
rno-miR-23b	ggtAatmCccTggmCaaTgtGat	76	38
rno-miR-24	cTgtTccTgcTgaActGagmCca	80	35
rno-miR-25	tcaGacmCgaGacAagTgcAatg	77	27
rno-miR-26a	gcmCtaTccTggAttActTgaa	70	34
rno-miR-26b	aacmCtaTccTgaAttActTgaa	65	28
rno-miR-27a	gcGgaActTagmCcamCtgTgaa	77	35
rno-miR-27b	gcAgaActTagmCcamCtgTgaa	74	38
rno-miR-28	ctmCaaTagActGtgAgcTccTt	73	43
rno-miR-290	aaaAagTgcmCccmCatAgtTtgAg	75	29
rno-miR-291-3p	gGcamCacAaaGtgGaaGcamCtt	78	52
rno-miR-291-5p	aGagAggGccTccActTtgAtg	77	46
rno-miR-292-3p	acActmCaaAacmCtgGcgGcamCtt	80	33
rno-miR-292-5p	caaAagAgcmCccmCagTttGagt	76	32
rno-miR-296	acAggAttGagGggGggmCcct	88	48

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
rno-miR-297	cAtgmCatAcaTgcAcamCatAcat	74	47
rno-miR-298	gGaaGaamCagmCccTccTctGcc	82	53
rno-miR-299	aTgtAtgTggGacGgtAaamCca	80	35
rno-miR-29a	aamCcgAttTcaGatGgtGcta	75	43
rno-miR-29b	aamCacTgaTttaCaaAtgGtgmCta	71	47
rno-miR-29c	amCcgAttTcaAatGgtGcta	71	47
rno-miR-300	gAagAgaGctTgcmCctTgcAta	77	35
rno-miR-301	atGctTtgAcaAtamCtaTtgmCacTg	72	42
rno-miR-30a-3p	gctGcaAacAtcmCgamCtgAaag	74	28
rno-miR-30a-5p	cTtcmCagTcgAggAtgTttAca	73	31
rno-miR-30b	agcTgaGtgTagGatGttTaca	71	33
rno-miR-30c	gmCtgAgaGtgTagGatGttTaca	73	33
rno-miR-30d	cttmCcaGtcGggGatGttTaca	76	44
rno-miR-30e	tcmCagTcaAggAtgTttAca	69	30
rno-miR-31	cagmCtaTgcmCagmCatmCttGcct	79	38
rno-miR-32	gcaActTagTaaTgtGcaAta	65	43
rno-miR-320	tTcgmCccTctmCaamCccAgtTtt	80	26
rno-miR-322	tgtTgcAgtGctTcaTgtTt	74	48
rno-miR-323	agAggTcgAccGtgTaaTgtGc	80	46
rno-miR-324-3p	ccAgtAgtAccTggGgcAgtGg	92	41
rno-miR-324-5p	acAccAatGccmCtaGggGatGcg	84	54
rno-miR-325	acamCttActGagmCacmCtamCtaGg	78	42
rno-miR-326	actGgaGgaAggGccmCagAgg	86	46
rno-miR-327	accmCtcAtgmCccmCtcAagg	76	27
rno-miR-328	acGgaAggGcaGagAggGccAg	87	31
rno-miR-329	aAaaAggTtaGctGggTgtGtt	75	32
rno-miR-33	cAatGcaActAcaAtgmCac	68	30
rno-miR-330	tmCtcTgcAggmCccTgtGctTtgc	83	52
rno-miR-331	tTctAggAtaGgcmCcaGggGc	84	51
rno-miR-333	aaaAgtAacTagmCacAccAc	69	24
rno-miR-335	amCatTttTcgTtaTtgmCtcTtga	67	26
rno-miR-336	aGacTagAtaTggAagGgtGa	75	28
rno-miR-337	aaaGgcAtcAtaTagGagmCtgAa	74	35
rno-miR-338	tcaAcaAaaTcamCtgAtgmCtgGa	73	33
rno-miR-339	tgAgcTccTggAggAcaGgga	83	47

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
rno-miR-340	ggcTatAaaGtaActGagAcgGa	72	34
rno-miR-341	amCtgAccGacmCgacGtcGa	84	53
rno-miR-342	gacGggTgcGatTtcTgtGtgAga	82	34
rno-miR-343	tctGggmCacAcgGagGgaGa	87	40
rno-miR-344	amCggTcaGgcTttGgcTagAtca	81	63
rno-miR-345	gcActGgacGtaGggGtcAgca	83	43
rno-miR-346	aGagGcaGgcActmCagGcaGaca	86	37
rno-miR-347	tggGcgAccmCagAggGaca	82	43
rno-miR-349	agaGgtTaaGacAgcAggGctg	79	39
rno-miR-34a	aamCaamCcaGctAagAcamCtgmCca	80	27
rno-miR-34b	caaTcaGctAatTacActGccTa	71	40
rno-miR-34c	gcAatmCagmCtaActAcamCtgmCct	76	31
rno-miR-350	gTgaAagTgtAtgGgcTttGtgAa	76	42
rno-miR-351	cagGctmCaaAggGctmCctmCagGga	84	59
rno-miR-352	tamCtaTgcAcacmCtamCtamCtct	68	26
rno-miR-421	caAcaAacAttTaaTgaGgcc	68	30
rno-miR-7	aacAaaAtcActAgtmCttmCca	66	30
rno-miR-7*	tatGgcAgamCtgTgaTttGttg	73	45
rno-miR-7b	aamCaaAatmCacAagTctTcca	68	24
rno-miR-9	tcAtamCagmCtaGatAcacmCaaAga	71	34
rno-miR-92	cagGccGggAcaAgtGcaAta	79	28
rno-miR-93	ctAccTgcAcgAacAgcActTtg	77	31
rno-miR-96	aGcaAaaAtgTgcTagTgcmCaaa	75	38
rno-miR-98	aAcaAtamCaamCttActAccTca	67	17
rno-miR-99a	cacAagAtcGgaTctAcgGtt	77	42
rno-miR-99b	cgcAagGtcGgtTctAcgGtg	82	42
zma-miR156a	gtgmCtcActmCtcTtcTgtmCa	71	25
zma-miR156b	gtgmCtcActmCtcTtcTgtmCa	71	25
zma-miR156c	gtgmCtcActmCtcTtcTgtmCa	71	25
zma-miR156d	gtgmCtcActmCtcTtcTgtmCa	71	25
zma-miR156e	gtgmCtcActmCtcTtcTgtmCa	71	25
zma-miR156f	gtgmCtcActmCtcTtcTgtmCa	71	25
zma-miR156g	gtgmCtcActmCtcTtcTgtmCa	71	25
zma-miR156h	gtgmCtcActmCtcTtcTgtmCa	71	25
zma-miR156i	gtgmCtcActmCtcTtcTgtmCa	71	25

Probe name	Probe sequence (5'-3')	Calc Tm °C	Self-complem. score
zma-miR160a	tggmCatAcaGggAgcmCagGca	85	49
zma-miR160b	tggmCatAcaGggAgcmCagGca	85	49
zma-miR160c	tggmCatAcaGggAgcmCagGca	85	49
zma-miR160d	tggmCatAcaGggAgcmCagGca	85	49
zma-miR160e	tggmCatAcaGggAgcmCagGca	85	49
zma-miR162	tggAtgmCagAggTttAtcGa	73	28
zma-miR164a	tgcAcgTgcmCctGctTctmCca	82	46
zma-miR164b	tgcAcgTgcmCctGctTctmCca	82	46
zma-miR164c	tgcAcgTgcmCctGctTctmCca	82	46
zma-miR164d	tgcAcgTgcmCctGctTctmCca	82	46
zma-miR166a	gGggAatGaaGccTggTccGa	84	33
zma-miR166b	gggAatGaaGccTggTccGa	79	29
zma-miR166c	gggAatGaaGccTggTccGa	79	29
zma-miR166d	gggAatGaaGccTggTccGa	79	29
zma-miR166e	gggAatGaaGccTggTccGa	79	29
zma-miR166f	gggAatGaaGccTggTccGa	79	29
zma-miR166g	gggAatGaaGccTggTccGa	79	29
zma-miR166h	gggAatGaaGccTggTccGa	79	29
zma-miR166i	gggAatGaaGccTggTccGa	79	29
zma-miR167a	tAgaTcaTgcTggmCagmCttmCa	79	53
zma-miR167b	tAgaTcaTgcTggmCagmCttmCa	79	53
zma-miR167c	tAgaTcaTgcTggmCagmCttmCa	79	53
zma-miR167d	tAgaTcaTgcTggmCagmCttmCa	79	53
zma-miR169a	tcGgcAagTcaTccTtgGctg	78	40
zma-miR169b	tcGgcAagTcaTccTtgGctg	78	40
zma-miR171a	ataTtgGcgmCggmCtcAatmCa	76	46
zma-miR171b	gtgAtaTtgGcamCggmCtcAa	74	43
zma-miR172a	tgmCagmCatmCatmCaaGatTct	73	39
zma-miR172b	tgmCagmCatmCatmCaaGatTct	73	39
zma-miR172c	tgmCagmCatmCatmCaaGatTct	73	39
zma-miR172d	tgmCagmCatmCatmCaaGatTct	73	39

## EXAMPLE 13

*Determination of microRNA expression in zebrafish embryonic development by whole mount in situ hybridization of embryos using LNA-substituted miRNA detection probes*

## Zebrafish

5 Zebrafish were kept under standard conditions (M. Westerfield, *The zebrafish book* (University of Oregon Press, 1993). Embryos were staged according to (C. B. Kimmel, W. W. Ballard, S. R. Kimmel, B. Ullmann, T. F. Schilling, *Dev Dyn* 203, 253-310 (1995). Homozygous albino embryos and larvae were used for the in situ hybridizations.

## LNA-substituted microRNA probes

10 The sequences of the LNA-substituted microRNA probes are listed below. The LNA probes were labeled with digoxigenin (DIG) using a DIG 3'-end labeling kit (Roche) and purified using Sephadex G25 MicroSpin columns (Amersham). For in situ hybridizations approximately 1-2 pmol of labeled probe was used.

**Table 1. List of LNA-substituted detection probes for determination of microRNA expression in zebrafish embryonic development by whole mount in situ hybridization of embryos**

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine.

Probe name	Probe sequence 5'-3'	Calc Tm °C
hsa-let7f/LNA	aamCtaTacAatmCtamCtamCctmCa	67
hsa-miR19b/LNA	tmCagTttTgcAtgGatTtgmCaca	75
hsa-miR17-5p/LNA	actAccTgcActGtaAgcActTtg	74
hsa-miR217/LNA	atcmCaaTcaGttmCctGatGcaGta	75
hsa-miR218/LNA	acAtgGttAgaTcaAgcAcaa	70
hsa-miR222/LNA	gaGacmCcaGtaGccAgaTgtAgct	80
hsa-let7i/LNA	agmCacAaamCtamCtamCctmCa	71
hsa-miR27b/LNA	cagAacTtaGccActGtgAa	68
hsa-miR301/LNA	gctTtgAcaAtamCtaTtgmCacTg	70
hsa-miR30b/LNA	gcTgaGtgTagGatGttaca	70
hsa-miR100/LNA	cacAagTtcGgaTctAcgGtt	77

Probe name	Probe sequence 5'-3'	Calc Tm °C
hsa-miR34a/LNA	aamCaamCcaGctAagAccmCta	80
hsa-miR7/LNA	aacAaaAtcActAgtmCttmCca	66
hsa-miR125b/LNA	tcamCaaGttAggGtcTcaGgga	77
hsa-miR133a/LNA	acAgcTggTtgAagGggAccAa	82
hsa-miR101/LNA	cttmCagTtaTcamCagTacTgta	68
hsa-miR108/LNA	aatGccmCctAaaAatmCctTat	66
hsa-miR107/LNA	tGatAgcmCctGtamCaaTgcTgct	80
hsa-miR153/LNA	tcamCttTtgTgamCtaTgcAa	68
hsa-miR10b/LNA	amCaaAttmCggTtcTacAggGta	73
mmu-miR10b/LNA	acacCaaAttmCggTtcTacAggg	73
hsa-miR194/LNA	tccAcaTggAgtTgcTgtTaca	75
hsa-miR199a/LNA	gaAcaGgtAgtmCtgAacActGgg	78
hsa-miR199a*/LNA	aacmCaaTgtGcaGacTacTgta	74
hsa-miR20/LNA	ctAccTgcActAtaAgcActTta	70
hsa-miR214/LNA	ctGccTgtmCtgTgcmCtgmCtgt	81
hsa-miR219/LNA	agAatTgcGttTggAcaAtca	70
hsa-miR223/LNA	gGggTatTtgAcaAacTgamCa	73
hsa-miR23a/LNA	gGaaAtcmCctGgcAatGtgAt	76
hsa-miR24/LNA	cTgtTccTgcTgaActGagmCca	80
hsa-miR26a/LNA	agcmCtaTccTggAttActTgaa	70
hsa-miR126/LNA	gcAttAttActmCacGgtAcga	71
hsa-miR126*/LNA	cgmCgtAccAaaAgtAatAatg	68
hsa-miR128a/LNA	aaAagAgamCcgGtmtCacTgtGa	77
mmu-miR7b/LNA	aamCaaAatmCacAagTctTcca	68
hsa-let7c/LNA	aamCcaTacAacmCtamCtamCctmCa	74
hsa-let7b/LNA	aamCcamCacAacmCtamCtamCctmCa	77
hsa-miR103/LNA	tmCatAgcmCctGtamCaaTgcTgct	80
hsa-miR129/LNA	agcAagmCccAgamCcgmCaaAaag	80
rno-miR129*/LNA	aTgcTttTtgGggTaaGggmCtt	78
hsa-miR130a/LNA	gcmCctTttAacAttGcamCtg	70
hsa-miR132/LNA	cgAccAtgGctGtaGacTgtTa	76
hsa-miR135a/LNA	tcamCatAggAatAaaAagmCcaTa	69
hsa-miR137/LNA	cTacGcgTatTctTaaGcaAta	68
hsa-miR200a/LNA	acaTcgTtamCcaGacAgtGtta	72
hsa-miR142-3p/LNA	tmCcaTaaAgtAggAaamCacTaca	72

Probe name	Probe sequence 5'-3'	Calc Tm °C
hsa-miR142-5p/LNA	gtaGtgmCttTctActTtaTg	63
hsa-miR181b/LNA	aamCccAccGacAgtGaaTgtt	81
hsa-miR183/LNA	caGtgAatTctAccAgtGccAta	73
hsa-miR190/LNA	acmCtaAtaTatmCaaAcaTatmCa	62
hsa-miR193/LNA	ctGggActTtgTagGccAggt	76
hsa-miR19a/LNA	tmCagTttTgcAtaGatTtgmCaca	72
hsa-miR204/LNA	cagGcaTagGatGacAaaGggAa	78
hsa-miR205/LNA	caGacTccGgtGgaAtgAagGa	81
hsa-miR216/LNA	camCagTtgmCcaGctGagAtta	74
hsa-miR221/LNA	gAaamCccAgcAgamCaaTgtAgct	80
hsa-miR25/LNA	tcaGacmCgaGacAagTgcAatg	77
hsa-miR29c/LNA	taamCcgAttTcaAatGgtGcta	70
hsa-miR29b/LNA	amCacTgaTttmCaaAtgGtgmCta	71
hsa-miR30c/LNA	gmCtgAgaGtgTagGatGttTaca	73
hsa-miR140/LNA	ctAccAtaGggTaaAacmCact	71
hsa-miR9*/LNA	acTttmCggTtaTctAgtTtta	65
hsa-miR92/LNA	amCagGccGggAcaAgtGcaAta	81
hsa-miR96/LNA	aGcaAaaAtgTgcTagTgcmCaaa	75
hsa-miR99a/LNA	cacAagAtcGgaTctAcgGgtt	77
hsa-miR145/LNA	aAggGatTccTggGaaAacTggAc	79
hsa-miR155/LNA	ccmCctAtcAcgAttAgtCttAa	71
hsa-miR29a/LNA	aamCcgAttTcaAatGgtGctAg	75
rno-miR140*/LNA	gtcmCgtGgtTctAccmCtgTggTa	81
hsa-miR206/LNA	ccamCacActTccTtamCatTcca	73
hsa-miR124a/LNA	tggmCatTcamCcgmCgtGccTtaa	80
hsa-miR122a/LNA	acAaamCacmCatTgtmCacActmCca	78
hsa-miR1/LNA	tamCatActTctTtamCatTcca	64
hsa-miR181a/LNA	acTcamCcgAcaGcgTtgAatGtt	77
hsa-miR10a/LNA	cAcaAatTcgGatmCtamCagGgtt	74
hsa-miR196a/LNA	ccaAcaAcaTgaAacTacmCta	67
hsa-let7a/LNA	aamCtaTacAacmCtamCtamCctmCa	70
hsa-miR9/LNA	tcAtamCagmCtaGatAacmCaaAga	71
hsa-miR210/LNA	agcmCgcTgtmCacAcgmCacAg	84
hsa-miR144/LNA	taGtamCatmCatmCtaTacTgtt	64
hsa-miR338/LNA	caAcaAaaTcamCtgAtgmCtgGa	72

Probe name	Probe sequence 5'-3'	Calc Tm °C
hsa-miR187/LNA	ggcTgcAacAcaAgamCacGa	79
hsa-miR200b/LNA	cAtcAttAccAggmCagTatTaga	71
hsa-miR184/LNA	cmCctTatmCagTtcTccGtcmCa	75
hsa-miR27a/LNA	gcGgaActTagmCcamCtgTgaa	77
hsa-miR215/LNA	ctgTcaAttmCatAggTcat	65
hsa-miR203/LNA	agTggTccTaaAcaTttmCac	68
hsa-miR16/LNA	ccaAtaTttAcgTgcTgcTa	68
hsa-miR152/LNA	aAgtTctGtcAtgmCacTga	72
hsa-miR138/LNA	gatTcamCaamCacmCagmCt	70
hsa-miR143/LNA	gagmCtamCagTgcTtcAtcTca	72
hsa-miR195/LNA	gmCcaAtaTttmCtgTgcTgcTa	73
hsa-mir375/LNA	tAacGcgAgcmCgaAcgAacAaa	79
dre-miR93/LNA	ctAccTgcAcaAacAgcActTt	73
dre-miR22/LNA	acaGttmCttmCagmCtgGcaGctt	76
dre-miR213/LNA	gGtamCagTcaAcgGtcGatGgt	80
dre-miR31/LNA	cagmCtaTgcmCaamCatmCttGcc	76
dre-miR189/LNA	amCtgTtaTcaGctmCagTagGcac	75
dre-miR18/LNA	tatmCtgmCacTaaAtgmCacmCtta	69
dre-miR15a/LNA	cAcaAacmCatTctGtgmCtgmCta	74
dre-miR34b/LNA	cAatmCagmCtaAcaAcamCtgmCcta	74
dre-miR148a/LNA	acaAagTtcTgtAatGcamCtga	69
dre-miR125a/LNA	camCagGttAagGgtmCtcAggGa	80
dre-miR139/LNA	agAcamCatGcamCtgTaga	69
dre-miR150/LNA	cacTggTacAagGatTggGaga	75
dre-miR192/LNA	ggcTgtmCaaTtcAtaGgtmCa	73
dre-miR98/LNA	aacAacAcaActTacTacmCtca	68
dre-let7g/LNA	amCtgTacAaamCaamCtamCctmCa	73
dre-miR30a-5p/LNA	gctTccAgtmCggGgaTgtTtamCa	80
dre-miR26b/LNA	aacmCtaTccTggAttActTgaa	68
dre-miR21/LNA	cAacAccAgtmCtgAtaAgcTa	72
dre-miR146/LNA	accmCttGgaAttmCagTtcTca	72
dre-miR182/LNA	tgtGagTtcTacmCatTgcmCaaa	72
dre-miR182*/LNA	taGttGgcAagTctAgaAcca	72
dre-miR220/LNA	aAgtGtcmCgaTacGgtTgtGg	81
hsa-miR138/LNA	gatTcamCaamCacmCagmCt	70

Probe name	Probe sequence 5'-3'	Calc Tm °C
dre-miR141/LNA	gcaTcgTtamCcaGacAgtGtt	74
hsa-miR143/LNA	gagmCtamCagTgcTtcAtcTca	72
hsa-miR195/LNA	gmCcaAtaTttmCtgTgcTgcTa	73
dre-mir-30a-3p/LNA	acaGcaAacAtcmCaamCtgAaag	72
hsa-mir375/LNA	tAacGcgAgcmCgaAcgAacAaa	79

#### Whole-mount in situ hybridizations

Whole-mount in situ hybridizations were performed essentially as described (B. Thisse et al., Methods Cell Biol 77, 505-19 (2004).), with the following modifications: Hybridization, washing and incubation steps were done in 2.0 ml eppendorf tubes. All PBS and SSC

5 solutions contained 0.1% Tween (PBST and SSCT). Embryos of 12, 16, 24, 48, 72 and 120 hpf were treated with proteinase K for 2, 5, 10, 30, 45 and 90 min, respectively. After proteinase K treatment and refixation with 4% paraformaldehyde, endogenous alkaline phosphatase activity was blocked by incubation of the embryos in 0.1 M ethanolamine and 2.5% acetic anhydride for 10 min, followed by extensive washing with PBST. Hybridizations

10 were performed in 200 µl of hybridization mix. The temperature of hybridization and subsequent washing steps was adjusted to approximately 22°C below the predicted melting temperatures of the LNA-modified probes. Staining with NBT/BCIP was done overnight at 4°C. After staining, the embryos were fixed overnight in 4% paraformaldehyde. Next, embryos were dehydrated in an increasing methanol series and subsequently placed in a 2:1

15 mixture of benzyl benzoate and benzyl alcohol. Embryos were mounted on a hollow glass slide and covered with a coverslip.

#### Plastic sectioning

Embryos and larvae stained by whole-mount in situ hybridization were transferred from benzyl benzoate/benzyl alcohol to 100% methanol and incubated for 10 min. Specimens

20 were washed twice with 100% ethanol for 10 min and incubated overnight in 100% Technovit 8100 infiltration solution (Kulzer) at 4°C. Next, specimens were transferred to a mold and embedded overnight in Technovit 8100 embedding medium (Kulzer) deprived of air at 4°C. Sections of 7 µm thickness were cut with a microtome (Reichert-Jung 2050), stretched on water and mounted on glass slides. Sections were dried overnight. Counterstaining was done

25 by 0.05% neutral red for 12 sec, followed by extensive washing with water. Sections were preserved with Pertex and mounted under a coverslip.

## Image acquisition

5 Embryos and larvae stained by whole-mount *in situ* hybridization were analyzed with Zeiss Axioplan and Leica MZFLIII microscopes and subsequently photographed with digital cameras. Sections were analyzed with a Nikon Eclipse E600 microscope and photographed with a digital camera (Nikon, DXM1200). Images were adjusted with Adobe Photoshop 7.0 software.

**Table 2. MicroRNA expression patterns in zebrafish embryonic development determined by whole mount *in situ* hybridization of embryos using LNA-substituted miRNA detection probes.**

MicroRNA	Class*	In situ expression pattern in zebrafish
miR-1	A	Body, head and fin muscles
miR-122a	A	Liver; pancreas
miR-124a	A	Differentiated cells of brain; spinal cord and eyes; cranial ganglia
miR-128a	A	Brain (specific neurons in fore- mid- and hindbrain); spinal cord; cranial nerves/ganglia
miR-133a	A	Body, head and fin muscles
miR-138	A	Outflow tract of the heart; brain; cranial nerves/ganglia; undefin. bilateral structure in headneurons in spinal cord
miR-144	A	Blood
miR-194	A	Gut and gall bladder; liver; pronephros
miR-206	A	Body, head and fin muscles
miR-219	A	Brain (mid- and hindbrain); spinal cord
miR-338	A	Lateral line; cranial ganglia
miR-9	A	Proliferating cells of brain, spinal cord and eyes
miR-9*	A	Proliferating cells of brain, spinal cord and eyes
miR-200a	A	Nose epithelium; lateral line organs; epidermis; gut (proctodeum); taste buds
miR-132	A	Brain (specific neurons in fore- and midbrain)
miR-142-5p	A	Thymic primordium
miR-7	A	Neurons in forebrain; diencephalon/hypothalamus; pancreatic islet
miR-143	A	Gut and gall bladder; swimbladder; heart; nose
miR-145	A	Gut and gall bladder; gills; swimbladder; branchial arches; fins; outflow tract of the heart; ear
miR-181a	A	Brain (tectum, telencephalon); eyes; thymic primordium; gills

<b>MicroRNA</b>	<b>Class*</b>	<b>In situ expression pattern in zebrafish</b>
miR-181b	A	Brain (tectum, telencephalon); eyes; thymic primordium; gills
miR-215	A	Gut and gall bladder
let-7a	A	Brain; spinal cord
let-7b	A	Brain; spinal cord
miR-125a	A	Brain; spinal cord; cranial ganglia
miR-125b	A	Brain; spinal cord; cranial ganglia
miR-142-3p	A	Thymic primordium; blood cells
miR-200b	A	Nose epithelium; lateral line organs; epidermis; gut (proctodeum); taste buds
miR-218	A	Brain (neurons and/or cranial nerves/ganglia in hindbrain); spinal cord
miR-222	A	Neurons and/or cranial ganglia in forebrain and midbrain; rhombomere in early stages
miR-23a	A	Pharyngeal arches; oral cavity; posterior tail; cardiac valves
miR-27a	A	Undefined structures in branchial arches; tip of tail in early stages
miR-34a	A	Brain (cerebellum); neurons in spinal cord
miR-375	A	Pituitary gland; pancreatic islet
miR-99a	A	Brain (hindbrain, diencephalon); spinal cord
let-7i	A	Brain (tectum, diencephalon)
miR-100	A	Brain (hindbrain, diencephalon); spinal cord
miR-103	A	Brain; spinal cord
miR-107	A	Brain; spinal cord
miR-126	A	Bloodvessels and heart
miR-137	A	Brain (neurons and/or cranial nerves/ganglia in fore-, mid- and hindbrain); spinal cord
miR-140	A	Cartilage of pharyngeal arches, head skeleton and fins
miR-140*	A	Cartilage of pharyngeal arches, head skeleton and fins
miR-141	A	Nose epithelium; lateral line organs; epidermis; gut (proctodeum); taste buds
miR-150	A	Cardiac valves; undefined structures in epithelium of branchial arches
miR-182	A	Nose epithelium; haircells of lateral line organs and ear; cranial ganglia; rods, cones and bipolar cells of eye; epiphysis
miR-183	A	Nose epithelium; haircells of lateral line organs and ear; cranial ganglia; rods, cones and bipolar cells of eye; epiphysis
miR-184	A	Lens; hatching gland in early stages

<b>MicroRNA</b>	<b>Class*</b>	<b>In situ expression pattern in zebrafish</b>
miR-199a	<b>A</b>	Epithelia surrounding cartilage of pharyngeal arches, oral cavity and pectoral fins; epidermis of head; tailbud
miR-199a*	<b>A</b>	Epithelia surrounding cartilage of pharyngeal arches, oral cavity and pectoral fins; epidermis of head; tailbud
miR-203	<b>A</b>	Most outer layer of epidermis
miR-204	<b>A</b>	Neural crest; pigment cells of skin and eye; swimbladder
miR-205	<b>A</b>	Epidermis; epithelia of branchial arches; intersegmental cells; not in sensory epithelia
miR-221	<b>A</b>	Brain (Neurons and/or cranial ganglia in forebrain and midbrain; rhombomere in early stages)
miR-7b	<b>A</b>	Brain (fore-, mid- and hindbrain); spinal cord
miR-96	<b>A</b>	Nose epithelium; haircells of lateral line organs and ear; cranial ganglia; rods, cones and bipolar cells of eye; epiphysis
miR-217	<b>B</b>	Brain (tectum, hindbrain); spinal cord; proliferative cells of eyes; pancreas
miR-126*	<b>B</b>	ND
miR-31	<b>B</b>	Ubiquitous
miR-216	<b>B</b>	Brain (tectum); spinal cord; proliferative cells of eyes; pancreas; body muscles
miR-30a-5p	<b>B</b>	Pronephros; cells in epidermis; lens in early stages
miR-153	<b>B</b>	Brain (fore- mid- and hindbrain, diencephalon/hypothalamus)
miR-15a	<b>C</b>	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-17-5p	<b>C</b>	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-18	<b>C</b>	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-195	<b>C</b>	Ubiquitous
miR-19b	<b>C</b>	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-20	<b>C</b>	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-26a	<b>C</b>	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-92	<b>C</b>	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
let-7c	<b>C</b>	Brain; spinal cord
miR-101	<b>C</b>	ND
miR-16	<b>C</b>	Brain
miR-21	<b>C</b>	Cardiac valves; otoliths in ear; rhombomere in early stages
miR-30b	<b>C</b>	Pronephros; cells in epidermis
miR-30c	<b>C</b>	Pronephros; cells in epidermis and epithelia of branchial arches; neurons in hindbrain
miR-26b	<b>C</b>	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)

<b>MicroRNA</b>	<b>Class*</b>	<b>In situ expression pattern in zebrafish</b>
let-7g	C	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-19a	C	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-210	C	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-22	C	Ubiquitous
miR-25	C	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-93	C	Ubiquitous (head, spinal cord, gut, outline somites, neuromasts)
miR-189	D	ND
miR-30a-3p	D	ND
miR-34b	D	Cells in pronephric duct; nose
miR-129*	D	ND
miR-135a	D	ND
miR-182*	D	ND
miR-187	D	ND
miR-220	D	ND
miR-301	D	ND
miR-223	D	ND
let-7f	-	Brain; spinal cord
miR-108	-	Ubiquitous
miR-IOa	-	Posterior trunk; later restricted to spinal cord
miR-IOb	-	Posterior trunk; later restricted to spinal cord
miR-129	-	Brain
miR-130a	-	ND
miR-139	-	Nose; neuromasts
miR-146	-	Neurons in forebrain; branchial arches and head skeleton
miR-148a	-	ND
miR-152	-	Ubiquitous
miR-155	-	ND
miR-190	-	ND
miR-193	-	ND
miR-196a	-	Posterior trunk; later restricted to spinal cord
miR-213	-	Nose (epithelium or olfactory neurons), eyes (ganglion cell layer)
miR-214	-	Epithelia surrounding cartilage of pharyngeal arches, oral cavity and pectoral fins; epidermis of head; tailbud
miR-24	-	Pharyngeal arches; oral cavity; posterior tail; cardiac valves
miR-27b	-	Cells in branchial arches
miR-29a	-	ND
miR-29b	-	ND

MicroRNA	Class*	In situ expression pattern in zebrafish
miR-29c	-	ND
miR-98	-	Brain

\* Main class in which expression patterns were compared: A, specific expression; B, marginal specific expression or very low absolute expression; C, ubiquitous expression. D, no detectable expression.

Wienholds *et al.*, Science, 2005, 309, 310-311 (published after the effective date of the data above) relates to the findings referred to in Table 2 - that reference also includes a number of figures which visually demonstrates the tissue distribution of a number of miRNAs. Wienholds *et al.* is consequently incorporated by reference herein.

5 **Table 3. List of LNA-substituted detection probes useful as specificity controls in detection of vertebrate microRNAs.**

LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine.

Probe name	Sequence 5'-3'	Self-comp score
hsa-miR206/LNA/2MM	ccamCacActmCtcTtamCatTcca	8
hsa-miR206/LNA/1MM	ccamCacActmCccTtamCatTcca	8
hsa-miR124a/LNA/2MM	tggmCatTcaAagmCgtGccTtaa	60
hsa-miR124a/LNA/1MM	tggmCatTcaAcgmCgtGccTtaa	60
hsa-miR122a/LNA/2MM	acAaamCacmCacmCgtmCacActmCca	18
hsa-miR122a/LNA/1MM	acAaamCacmCatmCgtmCacActmCca	18

10 The above demonstrates that it is possible to map an animal's miRNA against various tissues, and it is thus possible to determine the origin of a cell based on a determination of miRNA from said cell.

This has interesting implications. As mentioned above, it is a known clinical problem to determine the exact origin of a number of metastatic cancers and this has several consequences. First of all, it is not possible to locate the primary tumour (which may be 15 much smaller than the metastatic tumour which has been detected), but it is in such cases also difficult if not impossible to determine the optimum treatment because of lack of knowledge of the tissue origin of the primary tumour.

Cancer of unknown primary site is a common clinical entity, accounting for 2% of all cancer diagnoses in the Surveillance, Epidemiology, and End Results (SEER) registries between 1973 and 1987 (C. Muir. Cancer of unknown primary site *Cancer* 1995. 75: 353-356). In spite of the frequency of this syndrome, relatively little attention has been given to this group of 5 patients, and systematic study of the entity has lagged behind that of other areas in oncology. Widespread pessimism concerning the therapy and prognosis of these patients has been the major reason for the lack of effort in this area. The patient with carcinoma of unknown primary site is commonly stereotyped as an elderly, debilitated individual with metastases at multiple visceral sites. Early attempts at systemic therapy yielded low 10 response rates and had a negligible effect on survival, thereby strengthening arguments for a nihilistic approach to these patients. The heterogeneity of this group has also made the design of therapeutic studies difficult; it is well recognized that cancers with different biologies from many primary sites are represented. In the past 10 years, substantial 15 improvements have been made in the management and treatment of some patients with carcinoma of unknown primary site. The identification of treatable patients within this heterogeneous group has been made possible by the recognition of several clinical syndromes that predict chemotherapy responsiveness, and also by the development of specialized pathologic techniques that can aid in tumor characterization. Therefore, the optimal management of patients with cancer of unknown primary site now requires 20 appropriate clinical and pathologic evaluation to identify treatable subgroups, followed by the administration of specific therapy. Many patients with adenocarcinoma of unknown primary site have widespread metastases and poor performance status at the time of diagnosis. The outlook for most of these patients is poor, with median survival of 4 to 6 months. However, subsets of patients with a much more favorable outlook are contained within this large group, 25 and optimal initial evaluation enables the identification of these treatable subsets. In addition, empiric chemotherapy incorporating newer agents has produced higher response rates and probably improves the survival of patients with good performance status.

Fine-needle aspiration biopsy (FNA) provides adequate amounts of tissue for definitive diagnosis of poorly differentiated tumors, and identification of the primary source in about 30 one fourth of cases (CV. Reyes, K.S. Thompson, J.D. Jensen, and A.M. Chouelhury. Metastasis of unknown origin: the role of fine needle aspiration cytology *Diagn Cytopathol* 1998. 18: 319-322).

As one example, most patients with squamous cell carcinoma involving inguinal lymph nodes have a detectable primary site in the genital or anorectal area. In women, careful 35 examination of the vulva, vagina, and cervix is important, with biopsy of any suspicious areas. Men should undergo a careful inspection of the penis. Digital examination and anoscopy should be performed in both sexes to exclude lesions in the anorectal area.

Identification of a primary site in these patients is important, since curative therapy is available for carcinomas of the vulva, vagina, cervix, and anus even after they spread to regional lymph nodes. For the occasional patient in whom no primary site is identified, surgical resection with or without radiation therapy to the inguinal area sometimes results in long-term survival (A. Guarisch, TJ. Keane, and T. Elhakim. Metastatic inguinal nodes from an unknown primary neoplasm. A review of 56 cases *Cancer* 1987. 59: 572-577). Hence, clearly it is advantageous to be able to determine the origin of tumors and improved recognition of treatable subsets within the large heterogeneous population of patients with carcinoma of unknown primary site would represent a definite advance in the management and treatment of these patients. This will also allow treatable subsets to be defined with appropriate clinical and pathologic evaluation; Table X provides a summary of currently known subsets of carcinomas of unknown origin and outlines the recommended evaluation and treatment of. Clearly, identifying the primary site in cases of metastatic carcinoma of unknown origin has profound clinical importance in managing cancer patients. Currently, identification of the site of origin of a metastatic carcinoma is time consuming and often requires expensive whole-body imaging or invasive exploratory surgery.

Table X

Histopathology	Clinical Evaluation (in addition to history, Physical exam, routine laboratory, chest radiography)	Special Pathologic Studies	Specific Subsets for Therapy	Therapy
Adenocarcinoma (well-differentiated or moderately differentiated)	CT scan of abdomen Men serum PSA Women Mammograms Additional studies to evaluate signs, symptoms	Men PSA stain Women $\alpha$ ER, PE stain PSA or tumor PSA staining	1) Women, axillary node involvement 2) Women, peritoneal carcinomatosis 3) Men, blastic bone metastases, or high serum PSA or tumor PSA staining 4) Solitary metastatic lesion	Treat as primary breast cancer Treat as stage III prostate cancer Treat as stage IV prostate cancer Definitive local therapy
Squamous carcinoma	Cervical presentation Direct laryngoscopy, nasopharyngoscopy, bronchoscopy	—	Cervical adenopathy Inguinal adenopathy	Treat as locally advanced head/neck cancer Inguinal LJS(D $\pm$ radiation therapy
Poorly differentiated carcinoma	CT abdomen, chest Serum, HCG, AFP Additional studies to evaluate signs, symptoms	Immunoperoxidase staining, electron microscopy, cytogenetic studies	1) Features of EGCT 2) Other patients	Treat as nonseminomatous EGCT Empiric platinum or paclitaxel/platinum regimen
Neuroendocrine carcinoma	CT abdomen, chest Additional studies to evaluate signs, symptoms	Immunoperoxidase staining	1) Low grade 2) Small cell carcinoma 3) Poorly differentiated	Treat as advanced carcinoid tumor Empiric platinum/etoposide or platinum/etoposide/paclitaxel

CT = computed tomography, PSA = prostate specific antigen, HCG = human chorionic gonadotropin, AFP = alpha fetoprotein, ER = estrogen receptor, PR = progesterone receptor, EGCT = extragonadal germcell tumor, LND = lymph node dissection

As previously described, microRNAs have emerged as important non-coding RNAs, involved in a wide variety of regulatory functions during cell growth, development and differentiation. Some reports clearly indicate that microRNA expression may be indicative of cell differentiation state, which again is an indication of organ or tissue specification. This finding 5 has been confirmed in the experiments using LNA FISH probes on whole mount preparations in different developmental stages in zebra fish, where a large number of microRNAs display a very distinct tissue or organ-specific distribution. As outlined in the figures herein and in summary in table 2 many microRNAs are expressed only in single organs or tissues. For example, mir-122a is expressed primarily in liver and pancreas, mir-215 is expressed 10 primarily in gut and gall bladder, mir-204 is primarily expressed in the neural crest, in pigment cells of skin and eye and in the swimbladder, mir-142-5p in the thymic primordium etc. This catalogue of mir tissue expression profiles may serve as the basis for a diagnostic tool determining the tissue origin of tumors of unknown origin. If, for example a tumour sample from a given sample expresses a microRNA typical of another tissue type, this may 15 be predictive of the tumour origin. For example, if a lymph cancer type expresses microRNA markers characteristic of liver cells (eg. Mir-122a), this may be indicative that the primary tumour resides within the liver. Hence, the detailed microRNA expression pattern in zebrafish provided may serve as the basis for a diagnostic measurement of clinical tumour samples providing valuable information about tumour origin.

20 So, since it is possible to map miRNA in cells vs. the tissue origin of these cells, the present invention presents a convenient means for detection of tissue origin of such tumours.

Hence, the present invention in general relates to a method for determining tissue origin of tumours comprising probing cells of the tumour with a collection of probes which is capable of mapping miRNA to a tissue origin.

25 EXAMPLE 14

*Detection of microRNAs by in situ hybridization in paraffin-embedded mouse brain sections using 3' digoxigenin-labeled LNA probe*

A. Deparaffinization of the sections

30 (i) xylene 3x 5min, (ii) ethanol 100% for 2x 5min, ethanol 70% for 5min, ethanol 50% for 5min, ethanol 25% for 5min and in DEPC-treated water for 1min.

B. Deproteinization of sections

(i) 2x 5min in PBS; 5min in Proteinase K at 10ug/ml at 37°C (add Prot.K 20mg/ml to warm Prot.K buffer 20 min before incubation); 30sec in 0.2% Glycine in PBS and 2x30sec in PBS.

C. Fixation

Sections were fixed for 10 min in 4% PFA, and the slides rinsed 2x in PBS

5 D. Prehybridization

Prehybridization was carried out for 2 hours at the final hybridization temperature (ca 22 degrees below the predicted T<sub>m</sub> of the LNA probe) in hybridization buffer (50%Formamide, 5xSSC, 0.1%Tween, 9.2mM citric acid for adjustment to pH6, 50ug/ml heparin, 500ug/ml yeast RNA) in a humidified chamber (50% formamide, 5xSSC). Use DAKO Pen.

10 E. Hybridization

The 3' DIG-labeled LNA probe was diluted to 20 nM in hybridization buffer and 200ul of hybridization mixture was added per slide. The slides were hybridized overnight covered with Nescofilm in a humidified chamber. The slides were rinsed in 2x SCC and then washed at hybridization temperature 3 times 30 min in 50% formamide, 2xSSC, and finally 5x 5 min in

15 PBST at room temperature.

F. Immunological Detection

The slides were blocked for 1 hour in blocking buffer (2% sheep serum, 2mg/ml BSA in PBST) at room temperature, incubated overnight with anti-DIG antibody (1:2000 anti-DIG-AP Fab fragments in blocking buffer) in a humidified chamber at 4°C, washed 5-7 times 5 min in

20 PBST and 3 times 5 min in AP buffer (see below).

G. Colour reaction (room temperature, in dark)

The light-sensitive colour reaction (NBT/BCIP) was carried out for 1h-48h (400ul/slide) in a humidified chamber; the slides were washed for 3x 5 min in PBST, and mounted in aqueous mounting medium (glycerol) or dehydrate and mount in Entellan.

25 The results are shown in Figs. 5 and 6. It surprisingly appears that it is possible to detect target nucleotide sequences in these paraffin embedded sections. Previously it has been noted that it is very difficult to utilise fixated and embedded sections for hybridization assays.

This is due to a variety of factor: First of all, RNA is degraded over time, so the use of long hybridization probes to detect RNA becomes increasingly difficult over time. Secondly, the very structure of a fixated and embedded section is such that it appears to be difficult for hybridization probes to contact their target sequences.

5 Without being limited to any theory, it is believed that the short hybridization probes of the present invention overcome these disadvantages by being able to diffuse readily in a fixated and embedded section and by being able to hybridize with short fragments of degraded RNA still present in the section.

It should be noted that the present finding also opens for the possibility of detecting DNA in  
10 archived fixated and embedded samples. It is then e.g. possible, when using the short but highly specific probes of the present invention, to detect e.g. viral DNA in such aged samples, a possibility which to the best of the inventors' knowledge has not been available prior to the findings in the present invention.

H. Buffers used in example 14.

15 H1. AP buffer  
100ml Tris (100mM) 12.1g/l  
20ml 5M NaCl (100mM) 5.84g/l  
5ml 1M MgCl<sub>2</sub> (5mM)  
700ml sterile H<sub>2</sub>O, pH 9.5 and fill up to 1liter  
20 H2. Colour solution (Light sensitive)  
45ul 75mg/ml NBT (in 70% dimethylformamide)  
35ul 50mg/ml BCIP-phosphate (in 100% dimethylformamide)  
2.4mg Levamisole  
in 10 ml AP buffer.

## EXAMPLE 15

*Specificity and sensitivity assessment of microRNA detection in zebrafish, Xenopus laevis and mouse by whole mount *in situ* hybridization of embryos using LNA-substituted miRNA detection probes*

5 **Experimental material**

Zebrafish, mouse and Xenopus tropicalis were kept under standard conditions. For all *in situ* hybridizations on zebrafish we used 72 hour old homozygous albino embryos. For Xenopus tropicalis 3 day old embryos were used and for mouse we used 9.5 or 10.5 dpc embryos.

**Design and synthesis of LNA-modified oligonucleotide probes**

10 The LNA-modified DNA oligonucleotide probes are listed in Table 15-1. LNA probes were labeled with digoxigenin-ddUTP using the 3'-end labeling kit (Roche) according to the manufacturers recommendations and purified using sephadex G25 MicroSpin columns (Amersham).

15 **Table 15-1. List of short LNA-substituted detection probes for detection of microRNA expression in zebrafish by whole mount *in situ* hybridization of embryos**  
LNA nucleotides are depicted by capital letters, DNA nucleotides by lowercase letters, mC denotes LNA methyl-cytosine.

Probe name	Sequence 5'-3'	Calc Tm
hsa-miR124a/LNA	tggmCatTcamCcgmCgtGccTtaa	80
hsa-miR124a/LNA-2	gmCatTcamCcgmCgtGccTtaa	78
hsa-miR124a/LNA-4	atTcamCcgmCgtGccTtaa	72
hsa-miR124a/LNA-6	TcamCcgmCgtGccTtaa	71
hsa-miR124a/LNA-8	amCcgmCgtGccTtaa	70
hsa-miR124a/LNA-10	cgmCgtGccTtaa	60
hsa-miR124a/LNA-12	mCgtGccTtaa	46
hsa-miR124a/LNA-14	tGccTtaa	27
hsa-miR206/LNA	ccamCacActTccTtamCatTcca	73
hsa-miR206/LNA-2	amCacActTccTtamCatTcca	70
hsa-miR206/LNA-4	acActTccTtamCatTcca	64
hsa-miR206/LNA-6	ActTccTtamCatTcca	58

Probe name	Sequence 5'-3'	Calc Tm
hsa-miR206/LNA-8	tTccTtamCatTcca	55
hsa-miR206/LNA-10	ccTtamCatTcca	49
hsa-miR206/LNA-12	TtamCatTcca	35
hsa-miR206/LNA-14	amCatTcca	32
hsa-miR124a/LNA-8/MM	amCcgmCgtAccTtaa	70
hsa-miR206/LNA-8/MM	tTccTtaAatTcca	55

### Whole mount *in situ* hybridizations

All washing and incubation steps were performed in 2 ml eppendorf tubes. Embryos were fixed overnight at 4 °C in 4% paraformaldehyde in PBS and subsequently transferred through a graded series (25% MeOH in PBST (PBS containing 0.1% Tween-20), 50% MeOH in PBST, 5% MeOH in PBST) to 100% methanol and stored at -20 °C up to several months. At the first day of the *in situ* hybridization embryos were rehydrated by successive incubations for 5 min in 75% MeOH in PBST, 50% MeOH in PBST, 25% MeOH in PBST and 100% PBST (4 x 5 min). Fish, mouse and *Xenopus* embryos were treated with proteinaseK (10 µg/ml in PBST) for 45 min at 37 °C, refixed for 20 min in 4% paraformaldehyde in PBS and washed 3 x 5 min with PBST. After a short wash in water, endogenous alkaline phosphatase activity was blocked by incubation of the embryos in 0.1 M tri-ethanolamine and 2.5% acetic anhydride for 10 min, followed by a short wash in water and 5 x 5 min washing in PBST. The embryos were then transferred to hybridization buffer (50% Formamide, 5x SSC, 0.1% Tween, 9.2 mM citric acid, 50 µg/ml heparin, 500 µg/ml yeast RNA) for 2-3 hour at the hybridization temperature. Hybridization was performed in fresh pre-heated hybridization buffer containing 10 nM of labeled LNA probe. Post-hybridization washes were done at the hybridization temperature by successive incubations for 15 min in HM- (hybridization buffer without heparin and yeast RNA), 75% HM-/25% 2x SSCT (SSC containing 0.1% Tween-20), 50% HM-/50% 2x SSCT, 25% HM-/75% 2x SSCT, 100% 2x SSCT and 2 x 30 min in 0.2x SSCT. Subsequently, embryos were transferred to PBST through successive incubations for 10 min in 75% 0.2x SSCT/25% PBST, 50% 0.2x SSCT/50% PBST, 25% 0.2x SSCT/75% PBST and 100% PBST. After blocking for 1 hour in blocking buffer (2% sheep serum/2mg:ml BSA in PBST), the embryos were incubated overnight at 4 °C in blocking buffer containing anti-DIG-AP FAB fragments (Roche, 1/2000). The next day, zebrafish embryos were washed 6 x 15 min in PBST, mouse and *X. tropicalis* embryos were washed 6 x 1 hour in TBST containing 2 mM levamisole and then for 2 days at 4°C with regular refreshment of the wash buffer. After the post-antibody washes, the embryos were washed 3 x 5 min in staining buffer (100 mM tris HCl pH9.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.1% tween 20). Staining was done in buffer supplied with 4.5 µl/ml NBT (Roche, 50 mg/ml stock) and 3.5 µl/ml BCIP (Roche, 50 mg/ml stock).

stock). The reaction was stopped with 1 mM EDTA in PBST and the embryos were stored at 4°C. The embryos were mounted in Murray's solution (2:1 benzylbenzoate:benzylalcohol) via an increasing methanol series (25% MeOH in PBST, 50% MeOH in PBST, 75% MeOH in PBST, 100% MeOH) prior to imaging.

## 5 Image acquisition

Embryos and larvae stained by whole-mount *in situ* hybridization were analyzed with Zeiss Axioplan and Leica MZFLIII microscopes and subsequently photographed with digital cameras. Sections were analyzed with a Nikon Eclipse E600 microscope and photographed with a digital camera (Nikon, DXM1200). Images were adjusted with Adobe Photoshop 7.0

10 software.

## Results

We first compared the ability of LNA-modified DNA probes to detect miR-206, miR-124a and miR-122a in 72h zebrafish embryos with unmodified DNA probes of identical length and sequence. These three miRNAs are strongly expressed in the muscles, central nervous system and liver respectively. Both probe types could be easily labeled with digoxigenin (DIG) using standard 3' end labeling procedures. Labeling efficiency was checked by dot-blot analysis. Equal labeling was obtained for both LNA-modified and unmodified DNA probes (Fig. 15 7a). As depicted in Figure 7b, expected signals were obtained for all three miRNAs when LNA-modified probes were used for hybridization. In contrast, no such expression patterns could be seen with corresponding DNA probes under the same hybridization conditions. Lowering of 20 the hybridization temperature resulted in high background signals for all three DNA probes. Similar experiments to detect miRNAs in fish embryos using *in vitro* synthesized RNA probes, that carried a concatamer against the mature miRNA, were also unsuccessful. These results indicate that LNA-modified probes are well suited for sensitive *in situ* detection of miRNAs

## 25 Determination of the optimal hybridization temperature for LNA-modified probes

The introduction of LNA modifications in a DNA oligonucleotide probe increases the Tm value against complementary RNA with 2-10 °C per LNA monomer. Since the Tm values of LNA-modified probes can be calculated using a thermodynamic nearest neighbor model<sup>35</sup> we decided to determine the optimal hybridization temperature for detecting miRNAs in zebrafish 30 using LNA-modified probes, in relation to their Tm values (Table 15-1). The probes for miR-122a (liver specific) and miR-206 (muscle specific) have a calculated Tm value of 78 °C and 73 °C respectively. For miR-122a an optimal signal was obtained at a hybridization temperature of 58 °C and the probe for miR-206 gave the best signal at a temperature of 54

<sup>0</sup>C (Fig. 8a). A decrease or an increase in the hybridization temperature results in either higher background staining or complete loss of the hybridization signal. Thus, optimal results are obtained with hybridization temperatures of ~21-22 <sup>0</sup>C below the predicted Tm value of the LNA probe.

5 Apart from adjusting the hybridization temperature, standard *in situ* procedures also make use of higher formamide concentrations to increase the hybridization stringency. We used a formamide concentration of 50% and did not investigate the effects of formamide concentration on LNA-based miRNA *in situ* detection further, as the hybridization temperatures were in a convenient range.

10 **Determination of the optimal hybridization time for LNA-modified probes**

The standard zebrafish *in situ* protocol requires overnight hybridization. This may be necessary for long riboprobes used for mRNA *in situ* hybridization. We investigated the optimal hybridization time for LNA-based miRNA *in situ* hybridization. Significant *in situ* staining was obtained even after ten minutes of hybridization for miR-122a and miR-206 in 15 72 hour fish embryos (Fig. 8b). After one hour of hybridization the signal strength was comparable to the staining obtained after an overnight hybridization. This indicates that the hybridization times can be easily shortened for *in situ*s using LNA probes, which would reduce the overall miRNA *in situ* protocol for zebrafish from three to two days.

**Determination of the specificity of LNA-modified probes**

20 Many miRNAs belong to miRNA families. Some of the family members differ by one or two bases only, e.g. let-7c and let-7e (two mismatches) or miR-10a and miR-10b (one mismatch) and it might be that these do not have identical expression patterns. Indeed, from recent work it is clear that let-7c and let-7e have different expression patterns in the limb buds of the early mouse embryo. To examine the specificity of LNA-modified probes we set out to 25 perform *in situ* hybridizations with single and double mismatched probes for miR-124a, miR-206 and miR-122a (Table 15-1) under the same hybridization conditions as the fully complementary probe (Fig. 9). For miR-122a and miR-206 specific staining was lost upon introduction of a single central mismatch in the LNA probe. For the miR-124a probe two central mismatches were needed for adequate discrimination. These data demonstrate the 30 high specificity of LNA-based miRNA *in situ* hybridization.

To investigate if the *in situ* signal is fully coming from mature miRNAs or also from precursors, we designed probes against star and loop sequences of miR-183 and miR-217. miR-183 is specific for the haircells of the lateral line organ and the ear, rods and cones and

bipolar cells in the eye and sensory epithelia in the nose, while miR-217 is specific for the exocrine pancreas. We could not detect any pattern with probes against star and loop sequences for these miRNAs, suggesting that LNA-modified probes mainly detect mature miRNAs.

5 **Reduction of the LNA probe length**

In our initial *in situ* miRNA detection experiments, we used LNA-modified probes complementary to the complete mature miRNA sequence. Next, we decided to determine the minimal probe length, by which it would still be possible to get specific staining. Therefore, we systematically shortened the probes against miR-124a and miR-206 and performed *in situ* 10 hybridization on 72h zebrafish embryos with hybridization temperatures adjusted to 21 °C below the  $T_m$  value of the shortened probes. We could specifically detect miR-206 and miR-124a with shortened versions of the LNA probes complementary to a 12-nt region at the 5'-end of the miRNA (Fig. 10). *In situ* staining was virtually lost when 10-nt or 8-nt probes were used, although the 10-nt miR-124a probe gave a weak hybridization signal in the brain.

15 We expect that shorter LNA probes would exhibit significantly enhanced mismatch discrimination. As described above, in the case of miR-124a a single mismatch in a 22-mer LNA-modified probe was not sufficient for adequate discrimination. We thus tested single mismatch versions of the 14-mer LNA probes for miR-206 and miR-124a and found that in both cases the hybridization signal was completely lost (Fig. 10).

20 **Detection of miRNAs in *Xenopus laevis* and mouse embryos**

Thus far, we have reported the use of LNA probes for the detection of miRNAs only in the zebrafish embryo. To explore the usefulness of the LNA probe technology for detection of miRNAs in other organisms, we performed whole mount *in situ* hybridization on mouse and *Xenopus tropicalis* embryos with probes for miR-124a and miR-1, both of which are known to 25 be abundant and tissue specific miRNAs (Fig. 11a and b). miR-124a was specific for tissues of the central nervous system in both organisms. miR-1 was expressed in the body wall muscles and the muscles of the head in *Xenopus*. In mouse, miR-1 was mainly expressed in the somitic muscles and the heart. These data are in agreement with the expression patterns in zebrafish and with expression studies based on dissected tissues from mouse, which show 30 that miR-124a is brain specific and miR-1 is a muscle specific miRNA. Recently, a LacZ fusion construct of miR-1 also demonstrated that miR-1 is expressed in the heart and the somites of the early mouse embryo.

Next, we decided to determine the whole mount expression patterns in mouse embryos for miR-1, miR-206, miR-17, miR-20, miR-124a, miR-9, miR-126, miR-219, miR-196a, miR-10b and miR-10a, where the patterns were similar to what we previously observed in the zebrafish. In addition, miR-10a and miR-196a were found to be active in the posterior trunk 5 in mouse embryos as visualized by miRNA-responsive sensors and we also found these miRNAs to be expressed in the same regions. For miR-182, miR-96, miR-183 and miR-125b the expression patterns were different compared to zebrafish. miR-182, miR-96 and miR-183 are expressed in the cranial and dorsal root ganglia. In zebrafish the same miRNAs show expression in the haircells of the lateral line neuromasts and the inner ear but also in the 10 cranial ganglia. miR-125b is expressed at the midbrain hindbrain boundary in the early mouse embryo, whereas in zebrafish this miRNA is expressed in the brain and spinal cord.

Hence, based on the above it can be concluded that the present invention relates to aspects including:

- a) Use of an oligonucleotide in the isolation, purification, amplification, detection, 15 identification, quantification, inhibition or capture of non-coding RNAs characterized in that the oligonucleotide contains a number of nucleoside analogues;
- b) the use of such an oligonucleotide wherein the non-coding RNAs are selected from microRNAs, in particular mature microRNAs;
- c) such uses as in a or b wherein the number of nucleoside analogue corresponds to from 20 to 40 % of the oligonucleotide;
- d) such uses as in a, b or c, wherein the nucleoside analogue is LNA;
- e) such uses as in a, b, c or d, wherein the oligonucleotide comprises nucleoside analogues inserted with regular spacing between said nucleoside analogues, e.g. at every second nucleotide position, every third nucleotide position, or every fourth nucleotide position;
- f) such uses as in a, b, c, d or e in miRNA *in situ* hybridisation, dot blot hybridisation, reverse dot blot hybridisation, in expression profiling by oligonucleotide arrays or in Northern blot analysis;
- 25 g) such uses as in a, b, c, d or e in miRNA inhibition for functional analysis and antisense-based intervention against tumorigenic miRNAs and other non-coding RNAs;
- h) such uses as in a, b, c, d or e in miRNA detection for the identification of the primary site of metastatic tumors of unknown origin;
- i) such uses as in a, b, c, d, e, f, g, and h wherein the length of the oligonucleotide is less than about 21 nucleotides in length and more preferably less than 18 nucleotides, and most preferably between 12 and 14 nucleotides in length, and
- 30 j) a kit for the isolation, purification, amplification, detection, identification, quantification, or capture of a non-coding RNA, in particular mature microRNAs, the kit comprising a reaction body and one or more modified nucleotides.

## CLAIMS

1. A collection of detection probes, wherein each member of said collection comprises a recognition sequence consisting of nucleobases and affinity enhancing nucleobase analogues, and wherein the recognition sequences exhibit a combination of high melting temperatures and low self-complementarity scores, said melting temperatures being the melting temperature of the duplex between the recognition sequence and its complementary DNA or RNA sequence.  
5
2. The collection according to claim 1, wherein at least 80% of the detection probes include recognition sequences which exhibit a melting temperature or a measure of melting temperature corresponding to at least 5°C higher than a melting temperature or a measure of melting temperature of the self-complementarity score under conditions where the probe hybridizes specifically to its complementary target sequence.  
10
3. The collection according to claim 2, wherein at least 90% of the detection probes include recognition sequences which exhibit a melting temperature or a measure of melting temperature corresponding to at least 5°C higher than a melting temperature or a measure of melting temperature of the self-complementarity score under conditions where the probe hybridizes specifically to its complementary target sequence.  
15
4. The collection according to claim 2, wherein at least 95% of the detection probes include recognition sequences which exhibit a melting temperature or a measure of melting temperature corresponding to at least 5°C higher than a melting temperature or a measure of melting temperature of the self-complementarity score under conditions where the probe hybridizes specifically to its complementary target sequence.  
20
5. The collection according to claim 2, wherein all of the detection probes include recognition sequences which exhibit a melting temperature or a measure of melting temperature corresponding to at least 5°C higher than a melting temperature or a measure of melting temperature of the self-complementarity score under conditions where the probe hybridizes specifically to its complementary target sequence.  
25
6. The collection according to any one of the preceding claims, wherein the melting temperature or the measure of melting temperature is at least 10°C, such as at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, and at least 50°C higher than a melting temperature or measure of melting temperature to the self-complementarity score.  
30

7. The collection according to any one of the preceding claims, comprising at least 10 detection probes, 15 detection probes, such as at least 20, at least 25, at least 50, at least 75, at least 100, at least 200, at least 500, at least 1000, and at least 2000 members.
8. The collection according to any one of the preceding claims, which is capable of 5 specifically detecting all members of the transcriptome of an organism.
9. The collection according to any one of claims 1-8, which is capable of specifically detecting all small RNAs of an organism.
10. The collection according to claim 9, wherein the small RNAs are miRNA or siRNA.
11. The collection according to claim 9 or 10, wherein the organism is selected from the 10 group consisting of a bacterium, a yeast, a fungus, a protozoan, a plant, and an animal.
12. The collection according to any one of the preceding claims, wherein the affinity-enhancing nucleobase analogues are regularly spaced between the nucleobases in at least 80% of the members of said collection, such as in at least 90% or at least 95% of said collection.
13. The collection according to any one of the preceding claims, wherein the 3' and 5' 15 nucleobases are not substituted by affinity enhancing nucleobase analogues.
14. The collection according to any one of claims 1-13, wherein the presence of the affinity enhancing nucleobases in the recognition sequence confers an increase in the binding affinity between a probe and its complementary target nucleotide sequence relative to the binding 20 affinity exhibited by a corresponding probe, which only include nucleobases.
15. The collection according to any one of claims 1-14, wherein the affinity enhancing nucleobase analogues are LNA nucleobases.
16. The collection according to any one of the preceding claims, wherein the affinity enhancing nucleobase analogues are regularly spaced as every 2<sup>nd</sup>, every 3<sup>rd</sup>, every 4<sup>th</sup> or 25 every 5<sup>th</sup> nucleobase in the recognition sequence, preferably as every 3<sup>rd</sup> nucleobase.
17. The collection according to any one of the preceding claims, wherein the recognition sequence is at least a 6-mer, such as at least a 7-mer, at least an 8-mer, at least a 9-mer, at least a 10-mer, at least an 11-mer, at least a 12-mer, at least a 13-mer, at least a 14-mer,

at least a 15-mer, at least a 16-mer, at least a 17-mer, at least an 18-mer, at least a 19-mer, at least a 20-mer, at least a 21-mer, at least a 22-mer, at least a 23-mer, and at least a 24-mer.

18. The collection according to any one of claims 1-16, wherein the recognition sequence is at most a 25-mer, such as at most a 24-mer, at most a 23-mer, at most a 22-mer, at most a 21-mer, at most a 20-mer, at most a 19-mer, at most an 18-mer, at most a 17-mer, at most a 16-mer, at most a 15-mer, at most a 14-mer, at most a 13-mer, at most a 12-mer, at most an 11-mer, at most a 10-mer, at most a 9-mer, at most an 8-mer, at most a 7-mer, and at most a 6-mer.
19. The collection according to any one of the preceding claims, wherein at least 80% of the members comprise recognition sequences of the same length, such as at least 90% or at least 95%.
20. The collection according to claim 19, wherein all members contain affinity enhancing nucleobase analogues with the same regular spacing in the recognition sequences.
21. The collection according to any one of the preceding claims, wherein at least one of the nucleobases in the recognition sequence is substituted with its corresponding selectively binding complementary (SBC) nucleobase.
22. The collection according to any one of the preceding claims, wherein the nucleobases in the sequence are selected from ribonucleotides and deoxyribonucleotides.
23. The collection according to claim 22, wherein the recognition sequence consists of affinity enhancing nucleobase analogues together with either ribonucleotides or deoxyribonucleotides.
24. The collection according to any one of the preceding claims, wherein each member is covalently bonded to a solid support.
25. The collection according to claim 24, wherein the solid support is selected from a bead, a microarray, a chip, a strip, a chromatographic matrix, a microtiter plate, and a fiber.
26. The collection according to any one of the preceding claims, wherein each detection probe includes a detection moiety and/or a ligand, optionally in the recognition sequence.

27. The collection according to any one of the preceding claims, wherein each detection probe includes a photochemically active group, a thermochemically active group, a chelating group, a reporter group, or a ligand that facilitates the direct or indirect detection of the probe or the immobilisation of the oligonucleotide probe onto a solid support.

5 28. A detection probe which is a member of a collection according to any one of the preceding claims.

29. A detection probe including a recognition sequence selected from the LNA containing recognition sequences set forth in the tables A-K, 1, 3 and 15-1 herein.

30. A method for expanding or building a collection according to any one of claims 1-27,  
10 comprising  
A) defining a reference nucleotide sequence consisting of nucleobases, said reference nucleotide sequence being complementary to a target sequence for which the collection does not contain a detection probe,  
B) substituting the reference nucleotide sequence's nucleobases with affinity enhancing  
15 nucleobase analogues to provide a set of chimeric sequences wherein,  
C) determining usefulness of each of the chimeric sequences based on assessment of their ability to self-anneal and their melting temperature, and  
D) synthesizing and adding, to the collection, a probe comprising as its recognition sequence  
the chimeric sequence with the optimum combination of high melting temperature and low  
20 self-annealing.

31. The method according to claim 30, wherein step B includes provision of all possible chimeric sequences which include a particular set of affinity enhancing nucleobase analogues.

32. The method according to claim 30 or 31, wherein only chimeric sequences, wherein the affinity enhancing nucleobase analogues are regularly spaced between the nucleobases, are  
25 added to the collection in step D.

33. A method for designing an optimized detection probe for a target nucleotide sequence,  
comprising  
1) defining a reference nucleotide sequence consisting of nucleobases, said reference nucleotide sequence being complementary to said target nucleotide sequence,  
30 2) substituting the reference nucleotide sequence's nucleobases with affinity enhancing nucleobase analogues to provide a set of chimeric sequences  
3) determining usefulness of each of the chimeric sequences based on assessment of their ability to self-anneal and their melting temperatures, and

4) defining the optimized detection probe as the one in the set having as its recognition sequence the chimeric sequence with the optimum combination of high melting temperature and low self-annealing.

34. The method according to claim 33, wherein step 2 includes provision of all possible  
5 chimeric sequences which include a particular set of affinity enhancing nucleobase analogues.

35. The method according to claim 33 or 34, further comprising synthesizing the optimized detection probe.

36. The method according to any one of claims 33-35, wherein only chimeric sequences, wherein the affinity enhancing nucleobase analogues are regularly spaced between the  
10 nucleobases, are defined in step 4 or, if applicable, are synthesized.

37. The method according to any one of claims 33-36, wherein the detection probe is further modified by containing at least one SBC nucleobase as one of the nucleobases.

38. The method according to any one of claims 32-37, wherein the detection probe is a detection probe according to claim 28 or 29.

15 39. The method according to any one of claims 30-37, wherein, where applicable, steps A-C or 1-4, are performed in silico.

40. A computer system for designing an optimized detection probe for a target nucleic acid sequence, said system comprising  
a) input means for inputting the target nucleotide,  
20 b) storage means for storing the target nucleotide sequence,  
c) optionally executable code which can calculate a reference nucleotide sequence being complementary to said target nucleotide sequence and/or input means for inputting the reference nucleotide sequence,  
d) optionally storage means for storing the reference nucleotide sequence,  
25 e) executable code which can generate chimeric sequences from the reference nucleotide sequence or the target nucleic acid sequence, wherein said chimeric sequences comprise the reference nucleotide sequence, wherein has been in-substituted affinity enhancing nucleobase analogues,  
f) executable code which can determine the usefulness of such chimeric sequences based on  
30 assessment of their ability to self-anneal and their melting temperatures and either rank such chimeric sequences according to their usefulness,

g) storage means for storing at least one chimeric sequence, and  
h) output means for presenting the sequence of at least one optimized detection probe.

41. The computer system according to claim 40, wherein the target nucleic acid sequences are the sequences of non-coding small RNAs, such as miRNAs.

5 42. A computer system comprising executable code capable of executing the method according to claim 39.

43. Storage means comprising executable code which can execute the method steps according to claim 39.

10 44. A method for specific isolation, purification, amplification, detection, identification, quantification, inhibition or capture of a target nucleotide sequence in a sample, said method comprising contacting said sample with a member of a collection according to any one of claims 1-27 or with a probe according to claim 28 or 29 under conditions that facilitate hybridization between said member/probe and said target nucleotide sequence.

15 45. The method according to claim 44, used in isolation, purification, amplification, detection, identification, quantification, inhibition or capture of a molecule comprising the target nucleotide sequence.

46. The method according to claim 45, wherein the molecule is a small, non-coding RNA.

47. The method according to claim 46, wherein the molecule is miRNA such as a mature miRNA.

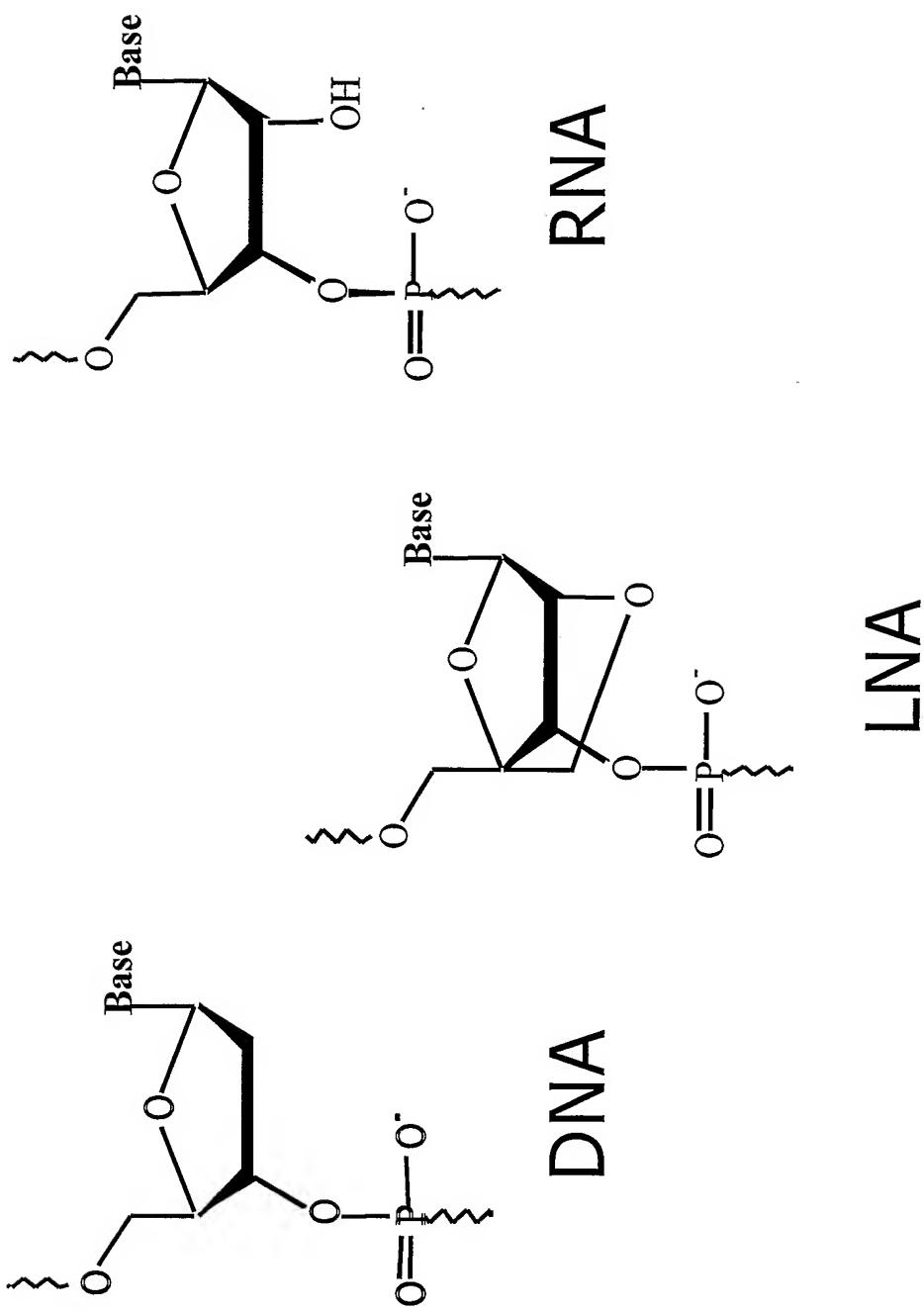
20 48. The method according to claim 47, used for the identification of the primary site of metastatic tumors of unknown origin.

49. The method according to any one of claims 45-48, wherein the small, non-coding RNA has a length of at most 30 residues, such as at most 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, or 18 residues.

25 50. The method according to any one of claims 45-48, wherein the small, non-coding RNA has a length of at least 15 residues, such as at least 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 residues.

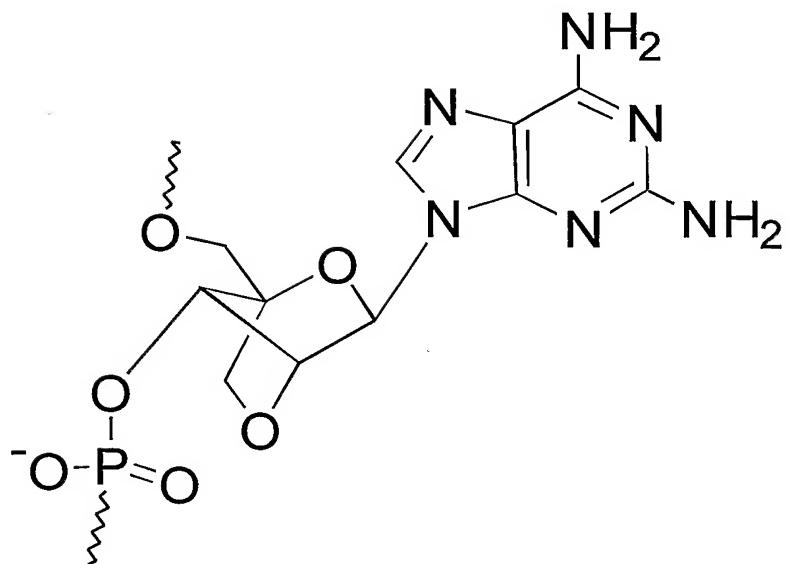
51. The method according to claim 45, wherein the molecule is DNA or RNA present in a fixated, embedded sample such as a formalin fixated paraffine embedded sample.
52. The method according to any one of claims 44-51, which is used in diagnosis, prognosis, therapy outcome prediction, and therapy.

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**Fig. 1**

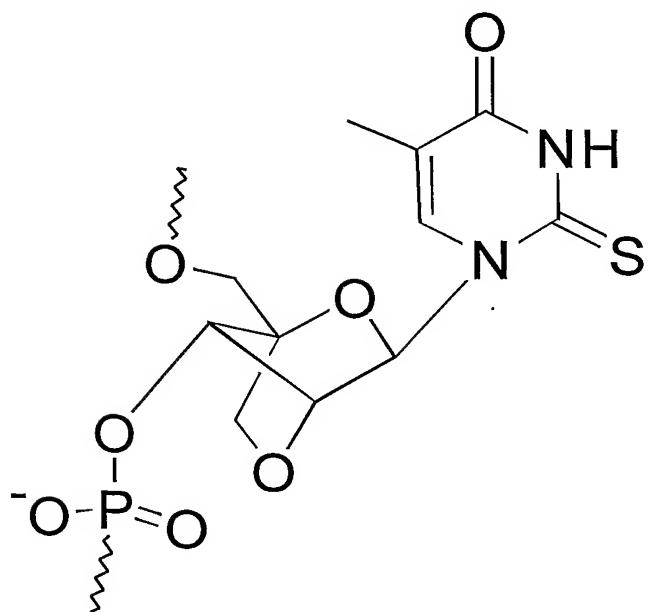
2/10  
**Fig. 2A**

**LNA-2,6-diaminopurine (LNA-D)**

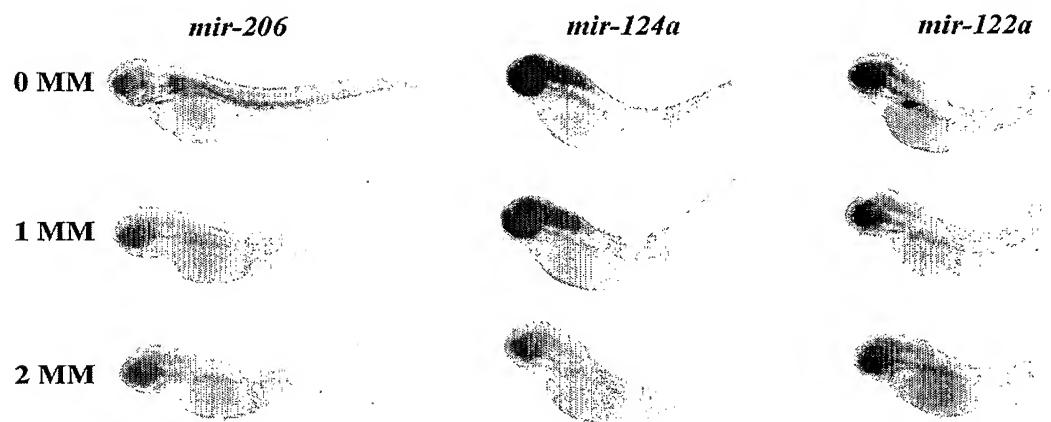


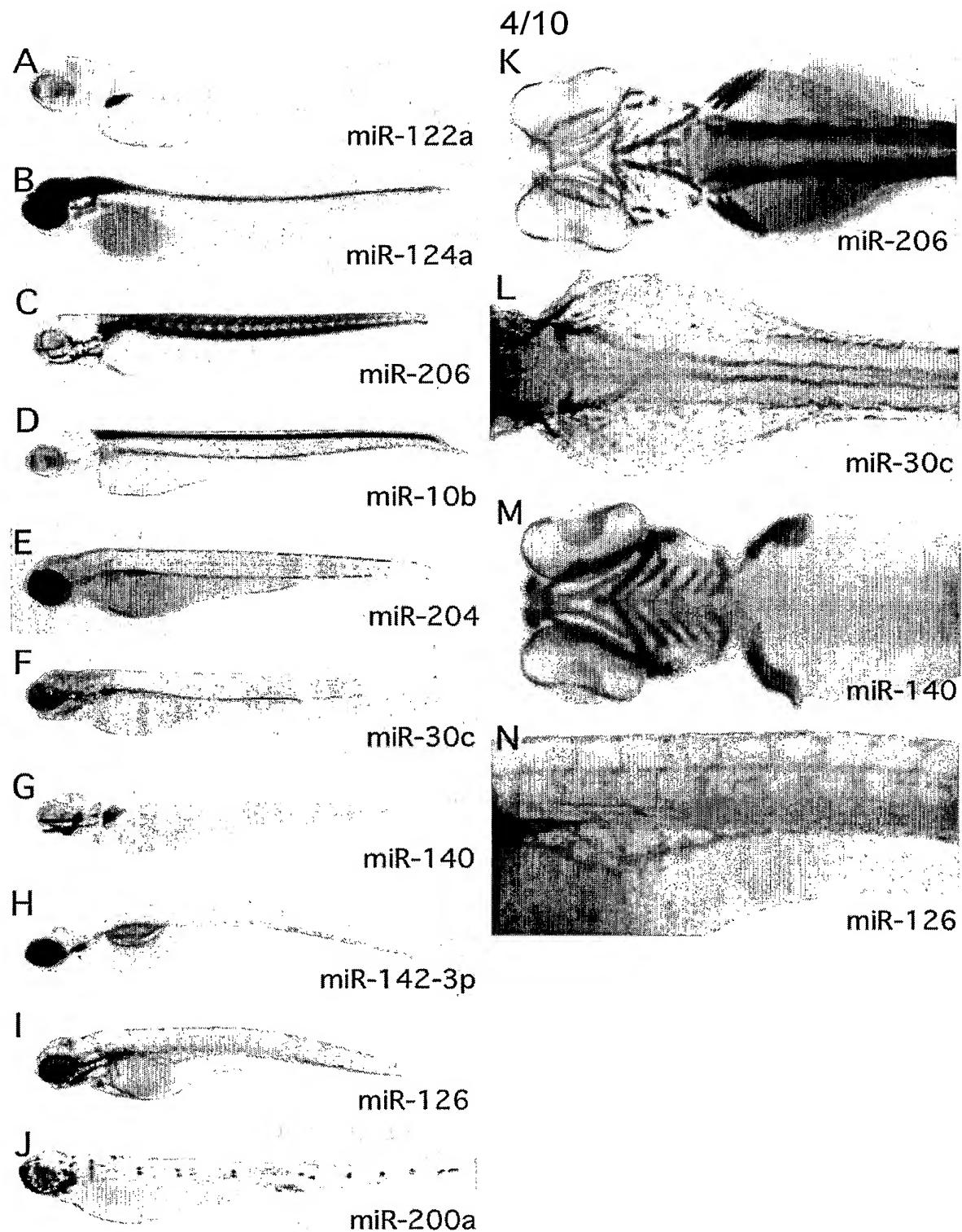
**Fig. 2B**

**LNA-2-thiothymidine (2-thio-T)**

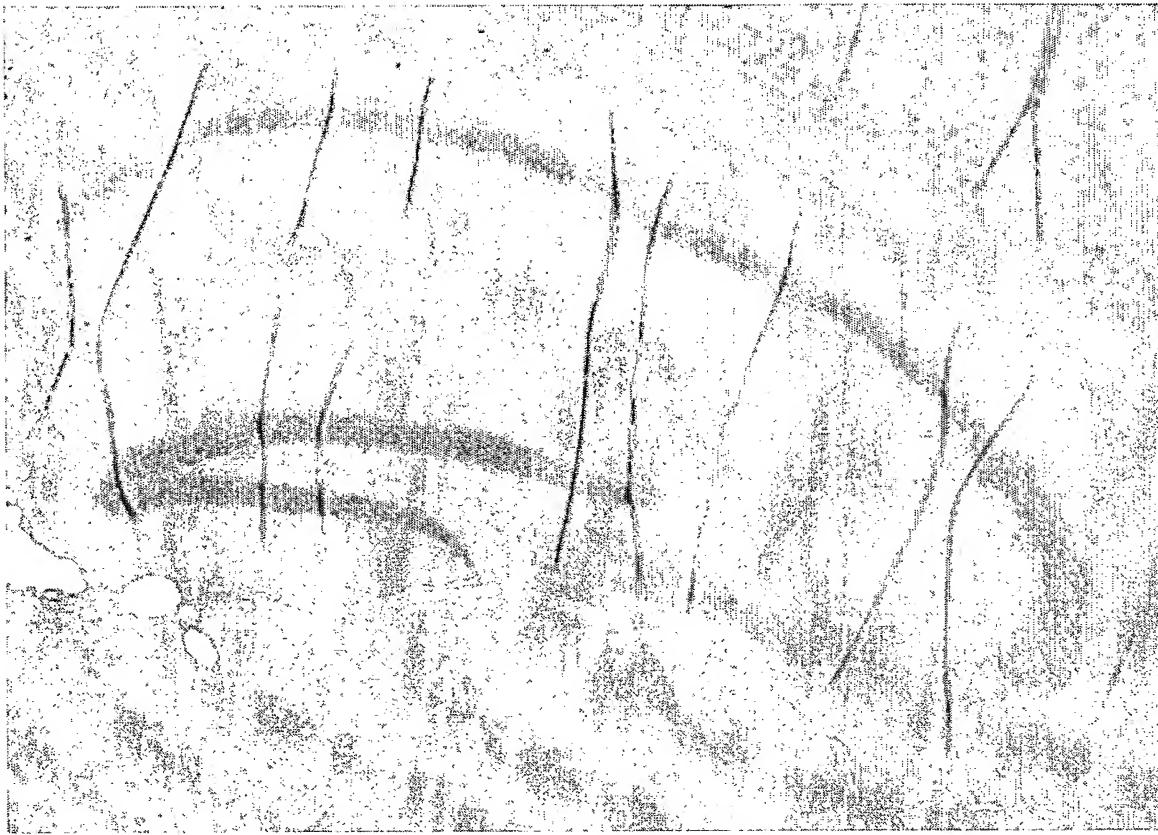


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**Fig. 3**

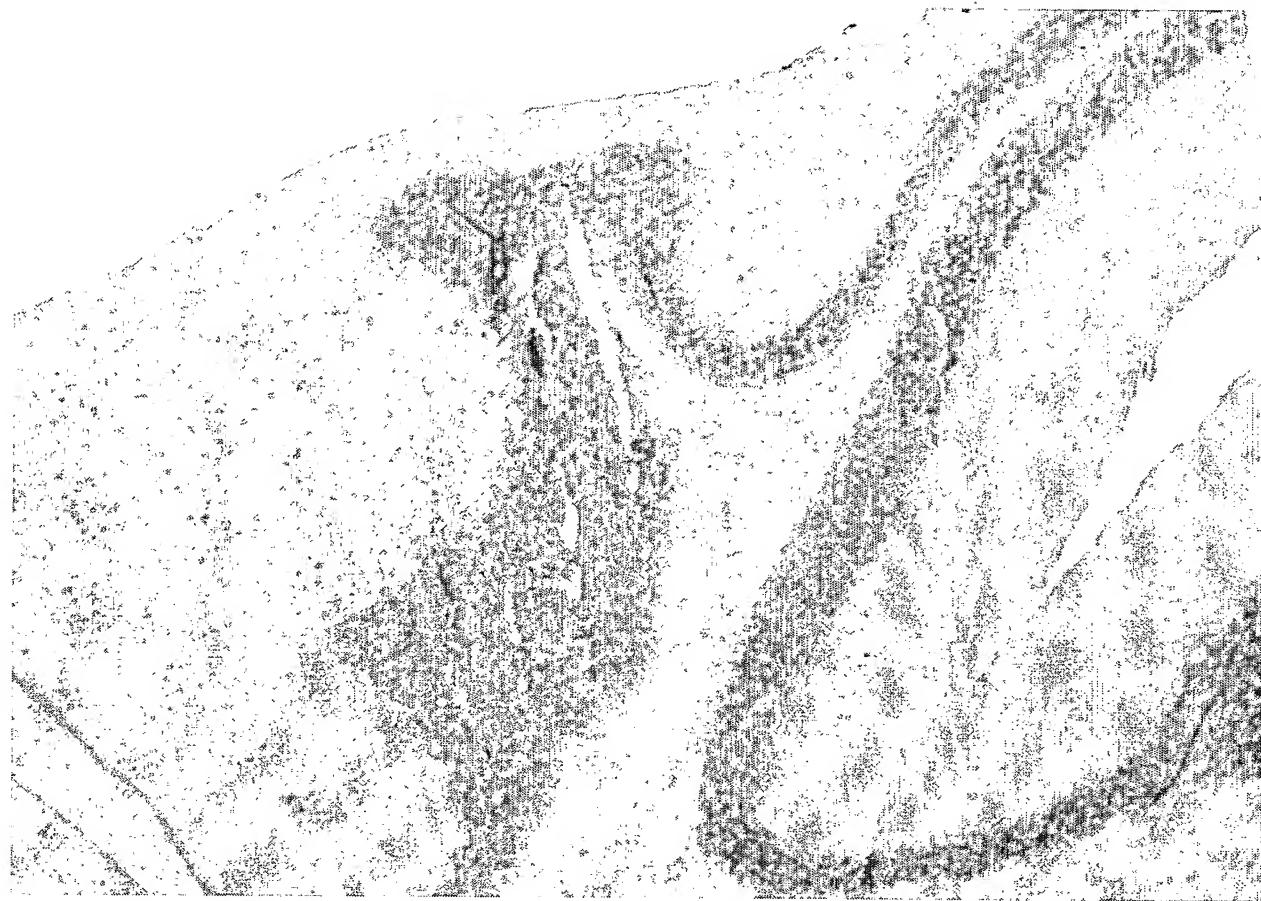
**Fig. 4**

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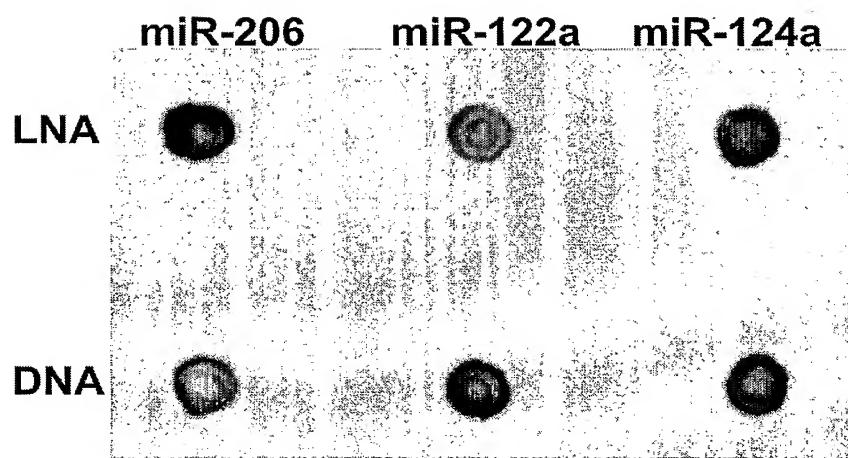
**Fig. 5**

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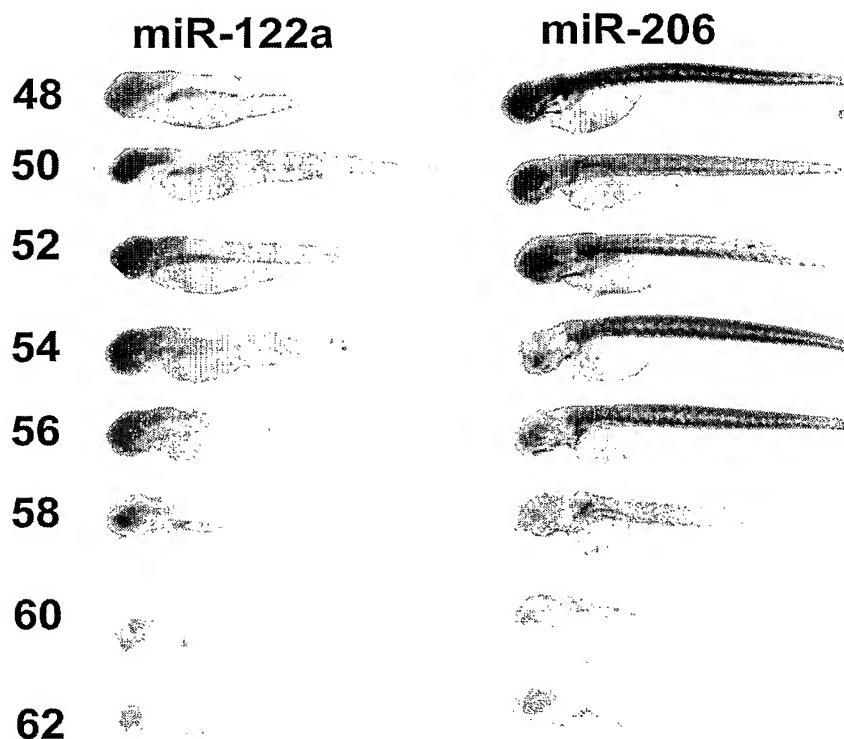


**Fig. 6**

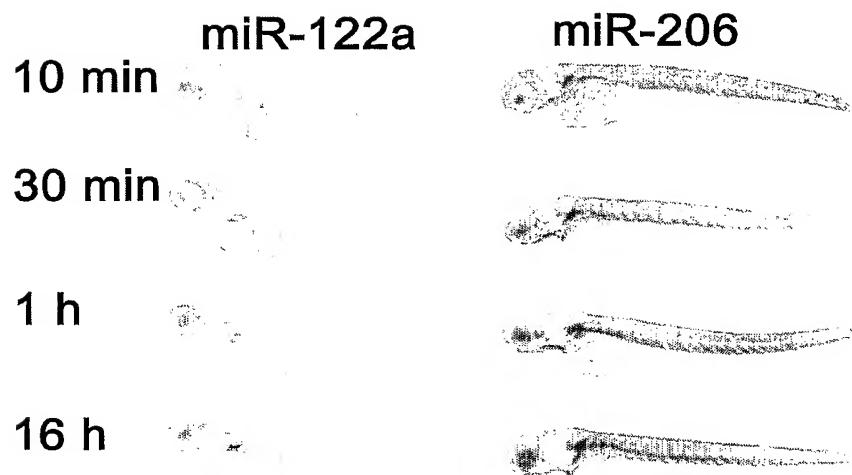
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**Fig. 7a****Fig. 7b**

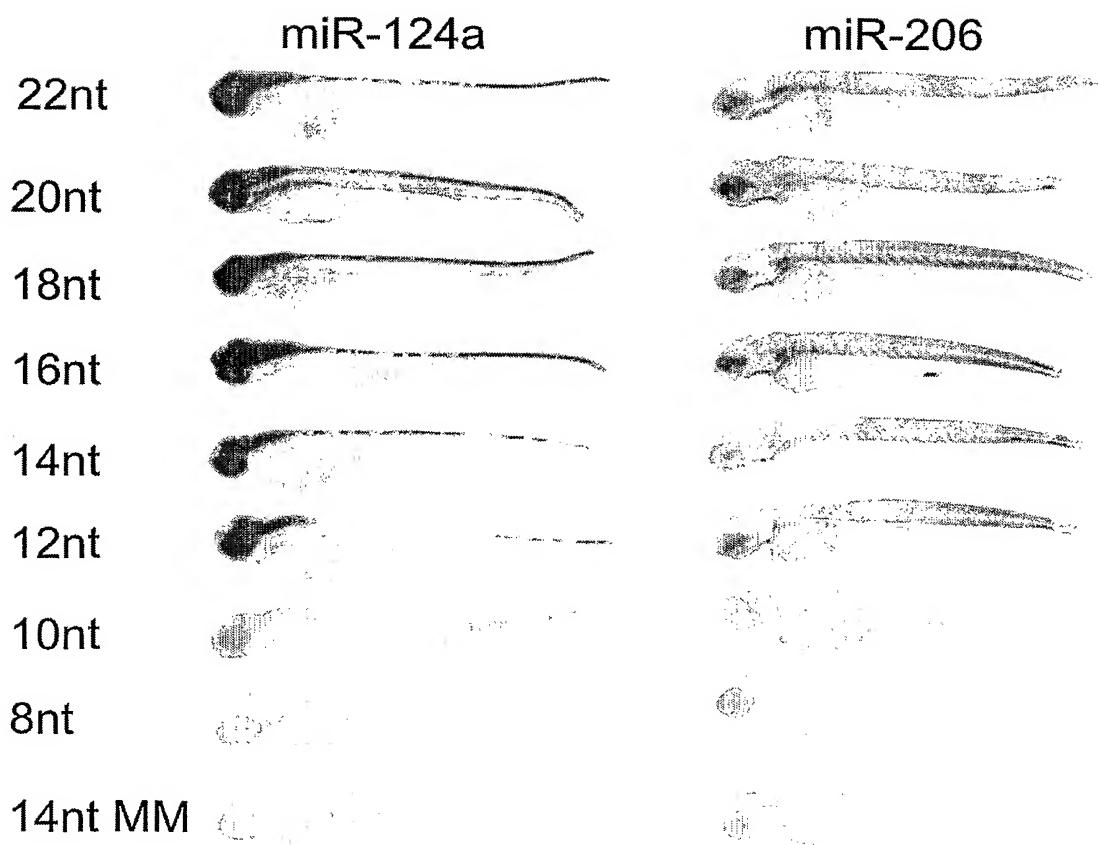
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**Fig. 8a**



**Fig. 8b**



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**Fig. 9****Fig. 10**

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**Fig. 11A**



**Fig. 11B**

